Regulation of HIF1 and IL-8 using RNAi induces Anti-Tumor Effects in Hepatocellular Carcinoma

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Directed by Professor Kwang-Hyub Han

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Sung Hoon Choi

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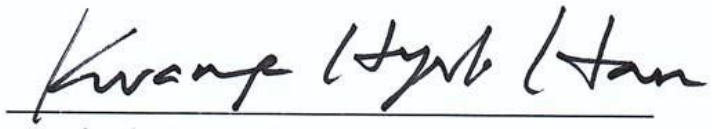
This certifies that the Doctoral Dissertation of Sung Hoon Choi is approved.



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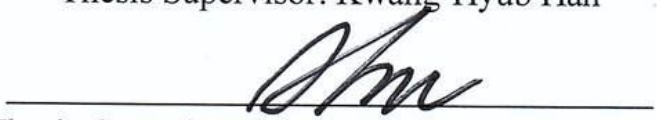

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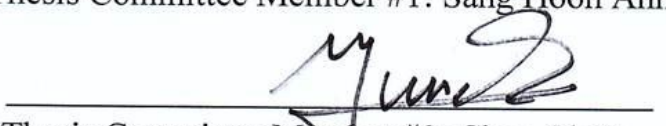

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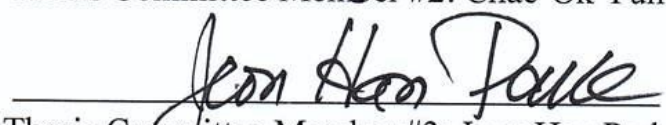

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ABSTRACT

Regulation of HIF1 and IL-8 using RNAi induces Anti-Tumor Effects in Hepatocellular Carcinoma

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Hypoxia is the condition where tumor cells have been deprived of oxygen and has been shown to have a role of tumor development in the hepatocellular carcinoma (HCC). The hypoxia inducible factors (HIFs) are transcriptional regulators that affect a homeostatic response to oxidative stress and have been identified as a key transcription activator of angiogenesis, survival, and metabolism. IL-8 also controlled endothelia cells survival and angiogenesis. Combination effects of HIF1α and IL-8 or HIF-1β were not fully supported by currently available evidences. In these studies, I evaluated the effects of HIFs and IL-8 knockdown on angiogenesis, apoptosis and tumor growth in HCC.

HCC cell lines were infected or transfected with adenoviruses expressing small-hairpin RNA (shRNA)/small interference RNA (siRNA) specific for HIFs or IL-8, cultured under hypoxic conditions (1% O_2), and examined for their levels of HIFs, IL-8, angiogenesis factors and apoptotic factors using immunoblot. The effects of adenovirus-mediated shRNA-induced HIF-1α and IL-8 knockdown on tumor growth and angiogenesis were also investigated in a

subcutaneous Hep3B-tumor xenograft mouse model. The expression levels of HIFs and apoptotic and growth factors were examined by real-time quantitative PCR and western blot. We also investigated apoptosis by TUNEL assay (FACS and immunofluorescence) and measured concentration of cytochrome C.

HIF-1α knockdown directly repressed tumor growth, whereas IL-8 knockdown indirectly repressed tumor growth. Combined knockdown of HIF-1α and IL-8 increased survival rates of mice. Conditioned media of Combined knockdown in HCC cells also decreased microvessel density and tumor volume *in vivo*. Similarly, HIF-1 α and IL-8 knockdown inhibited the angiogenic effects of HCC cell-conditioned media on tube formation and invasion by endothelial cells in vitro. Inhibition of HIF-1α and IL-8 up-regulated the expression of apoptotic factors while down-regulating anti-apoptotic factors simultaneously. Knockdown of HIF-1α and IL-8 increased concentration of cytosolic cytochrome C and enhanced DNA fragmentation in HCC cell lines and HUVECs. Moreover, culture supernatant collected from the knockdown of HIF-1α and IL-8 in HCC cell lines induced apoptosis in HUVECs under hypoxia. Silencing of HIF-1β expression suppressed tumor cell growth and inhibited the expression of tumor growth-related factors, such as vascular endothelial growth factor, epidermal growth factor, and hepatocyte growth factor. Suppression of tumor cell invasion and migration was also demonstrated in HIF-1β-silenced HCC cell lines.

These findings indicate that knockdown of HIFs and IL-8 inhibit angiogenesis, anti-apoptosis and tumor growth in HCC. Further development of HIFs and IL-8 shRNA/siRNA technologies could lead to effective therapies for HCC.

Key words: Hypoxia, Hepatocellular carcinoma, Angiogenesis, Cell survival, HIF-1α, IL-8, TUNEL, Tumor xenograft mouse model

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I. INTRODUCTION

Hepatocellular carcinoma(HCC) is the sixth most common cancer in the world. HCC is the second most lethal cancer. Most HCC developed hypoxic induction with angiogenesis and growth program[1].

The hypoxia inducible factors (HIFs) are a family of heterodimeric transcription factors that act as master regulators of a homeostatic transcriptional response to hypoxia in virtually all cells and tissues[1,2]. Active HIF consists of an alpha subunit and a beta subunit[2]. Three alpha subunits, termed `HIF1a, HIF2a, and HIF3a, have been described in humans, mice, and rats; all bind to a common b subunit named, alternatively, HIF1b, or the aryl-hydrocarbon-nuclear receptor translocator (ARNT)[3]. Active HIF is termed by its alpha subunit; hence, HIF1 is the active transcription factor consisting of HIF1α and ARNT, HIF2 is the dimer of HIF2α and ARNT, etc. HIF1 and HIF2 are the major hypoxia-inducible factors in humans, mice, and rats[3,4].

Under conditions of normoxia, HIF-1α subunits are hydroxylated at proline residues by hydroxylase enzymes[5]. Hydroxylation of HIF1α and

assembly on a protein scaffold consisting of the VHL tumor suppressor, along with other cofactors, result in the rapid ubiquitination of the alpha subunit and subsequent degradation by the proteasome. Conversely, in conditions of hypoxia, HIFα subunits escape degradation and are free to dimerize with the binding partner, ARNT[5,6]. The HIF trans-locates to the nucleus and affects transcription of target genes, typically by binding to a hypoxia response element (HRE) in the upstream promoter region of the target genes such as, angiogenesis, apoptosis, metabolism, survival etc[7].

Interlukine-8 (CXCL-8, IL-8) is a key factor of endothelial cell survival, angiogenesis. IL-8 is also regulated under hypoxia and directly controlled endothelial cell. IL-8 has been shown to regulate pathological angiogenesis, tumor growth, and metastasis[8]. The mechanism(s) regulating IL-8-mediated endothelial cell survival are not well understood. Recent reports suggest that in addition to cell proliferation and migration, endothelial cell survival and death are also important components for tumor survival and development[8,9]. Recent studies have shown that a cell cycle-regulated apoptosis inhibitor, survivin, and the cell death-related gene family products, Bcl-xl and Bcl-2, are associated with vascular endothelial growth factor (VEGF)-induced angiogenesis[10]. IL-8 and its receptors CXCR1 and CXCR2 have been observed in endothelial cells and have been shown to play a role in endothelial cell proliferation[9-11].

The most important factors that impact cancer progression are oxygen and nutrients. Large amounts of nutrients and oxygen are required for cancer cell proliferation, which further results in localized hypoxia[12]. This in turn causes tumor angiogenesis, which is the generation of new blood vessels from the already existing ones[12]. Tumor angiogenesis overcomes oxidative stress and deficiency of oxygen-dependent energy production caused by hypoxia[13]. The key factors responsible for the regulation of angiogenesis during hypoxia are HIF-1α and VEGF[14]. However, various studies have reported that angiogenesis is induced even during inhibition of HIF-1α during hypoxia, and these findings

demonstrate that tumor angiogenesis is also partially recovered by various other factors[14-16]. Increase in expression of various factors during hypoxia can induce angiogenesis, as they increase proliferation and ensure stabilization of endothelial cells, which are not only caused by HIF-1α, but also by cytokines such as interleukin IL-8, or other growth factors, such as PDGF[17]. They also increase VEGF expression as well as contribute to the increase and stabilization of angiogenesis by stimulation of VEGFR on the surface of endothelial cells[18]. Moreover, various solid tumors pass through the following 3 stages during their reproduction cycle: cell proliferation, hypoxia, and recovery by angiogenesis.

Majority of solid tumors are dependent on angiogenesis; therefore, angiogenesis inhibition can be used as a potential treatment modality to inhibit the proliferation and growth of solid tumors[19,20]. In addition, efforts to treat solid tumors using angiogenesis inhibitors have yielded good results[21]. However, these therapies not only affect solid tumors but also normal cells, which is an area of concern in cancer treatment. Furthermore, cancer therapies, such as transcatheter arterial chemoembolization (TACE) that uses blood vessels may not produce the desired results, and this may even increase vascular proliferation and growth into a malignant tumor by incomplete responses[22]. A correlation between hypoxia, cancer proliferation, and angiogenesis and the mechanism of growth or development of tumors has been observed[21-23]. If the link between any of the above can be elucidated, the basis for inhibition of tumor growth and excision can be ascertained[24]. This can be achieved by dual control of HIF-1α and angiogenic factors. Innovative and more effective cancer therapies can be developed by regulating HIF-1α expression, which is the key factor in hypoxia, and controlling the expression of IL-8 and other angiogenic stimulators, which restore the angiogenic processes, during inhibition of HIF-1α expression[25].

Inhibition of HIF-1α and IL-8 expression to suppress angiogenesis

Simultaneous inhibition of HIF-1α and IL-8 expression has proven to be more effective in hindering angiogenesis than inhibition of a single factor. With regards to expression at a molecular level, studies have demonstrated that liver cancer is regulated more by HIF-1α; however, in vascular endothelial cells, such as human umbilical vein endothelial cells (HUVEC), the level of IL-8 regulation is similar to that of HIF-1α. The growth of cancer cells and VEGF expression, which controls angiogenesis, have been observed to be regulated by HIF-1α, whereas IL-8 does not affect tumor growth and VEGF expression. Alternatively, in HUVEC, when IL-8 expression is inhibited, angiogenic inhibition is observed at a level similar to HIF-1α inhibition (Fig 1)[26,27].

Similar results have been obtained through animal research; inhibition of HIF-1α expression rarely resulted in tumors reproduction in the animal models. Moreover, no apoptosis of existing tumors are observed in these animals; however, in other animal models, where IL-8 expression was inhibited, a tumor volume similar to the time when shIL-8 was injected into the tumors was observed[27]. These findings did not reveal any correlation between IL-8 and direct growth of tumors; however, IL-8 plays an important role in angiogenesis. In addition, the results of the experiments on angiogenesis, such as invasion, tube formation, and aorta sprouting assays, have confirmed that simultaneous inhibition of two factors yielded more favorable responses than inhibition of a single factor[26,27]. Experiments with animal models have also demonstrated apoptosis of existing tumors as well as high survival rates in a majority of animals in which both the factors were inhibited. Moreover, it was confirmed that various factors to test for blood vessel formation, such as CD31, CD34, and vascular endothelial (V-E) cadherin, were not observed[25-27]. These findings suggested that controlling hypoxia as well as the expression of angiogenesis-associated factors that act via different pathways can aid in the

inhibition of angiogenesis[27].

Various treatments have been developed for cancer, and better therapies have been developed by overcoming the limitations of already developed treatments. I hypothesized that if the symptoms that occur during tumor treatment can be studied and controlled, the obstacles currently encountered during cancer treatment can be eliminated. If simultaneous regulation of tumor development, hypoxia, and angiogenesis is possible, cancer cells could be easily treated without peripheral damage. In other words, simultaneous inhibition of the factors that potentially control hypoxia and angiogenesis during treatment to induce apoptosis may be a more innovative anticancer treatment modality.

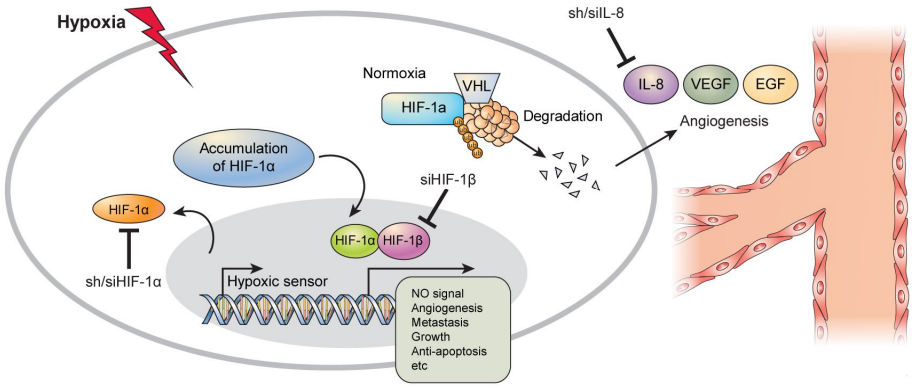


Figure 1. Inhibition of tumor angiogenesis by silencing of HIF-1α and IL-8. HIF-1α directly regulates HCC development and IL-8 assists tumor growth through regulation of angiogenesis in the vascular endothelial systems. shRNA-induced HIF-1α and IL-8 knockdown inhibit angiogenesis and tumor growth in HCC.

Tumor escape from apoptosis under hypoxic conditions

A correlation exists between cancer and hypoxia. Hypoxia during tumor development can destroy cancer cells; however, it acts as a key factor in excessive cancer proliferation[16]. Prevention of cancer by treatment of hypoxia can be used as a highly effective anticancer therapy. To date, studies have been carried out to induce apoptosis in various tumors. Reducing angiogenesis, typically by inhibiting VEGF, EGF, or bFGF, can be used as a treatment option, while the other involves activation of the intracellular intrinsic apoptosis pathway by inducing the expression of apoptotic factors and inhibiting the expression of anti-apoptosis factors[28]. Moreover, a method to stimulate an extracellular death signal outside cells in order to induce apoptosis can be developed[29].

Apoptosis, also called programmed cell death, is one of the most important cellular functions[29]. In normal cells, a decrease in telomere length occurs with age, or DNA damage, toxin exposure, and deprivation of growth factor generates death signals in various pathways, which results in apoptosis[30]. Hypoxic stimulation is also a crucial death signal; apoptosis is induced when the oxygen supply, required for production of ATP, an important cellular metabolite, is suppressed[31-33]. However, in tumors, stimulation that induces apoptosis can be avoided. During telomere length decrease, production of telomerase is promoted to restore the length of telomeres, and when DNA damage is induced, it can be repaired by mutation[34]. In hypoxic conditions, apoptosis can be avoided by inducing angiogenesis by increasing the expression of growth factors, thereby restoring oxygen supply, or by stimulating intracellular nitric oxide synthase (iNOs) through hematopoiesis and local vasodilation[35,36]. Apoptosis can be also be avoided by increasing anaerobic ATP production via glycolysis, which is facilitated by promotion GLUT1 or pyruvate dehydrogenase kinase activity[37].

HIF-1α, which is generated under hypoxic conditions, is an important

anti-apoptotic factor. HIF-1, like tumor necrosis factor (TNF)-a, activates the expression of FoxM1, that in turn induces growth of cancer cells in the liver and increases resistance to apoptosis[38,39]. The expression of HIF-1 in liver cancer inhibits expression of various caspases and reduces expression of Bax and Bak, which lead to a higher concentration of cytochrome C inside the cells. Increase in the expression of survivin and the Bcl-family[44], which are important factors that cause DNA fragmentation, can prevent hypoxic apoptosis[40,43].

Apoptosis in tumors is important because tumor angiogenesis, which is increased by tumor proliferation[45], can also be inhibited. Apoptosis in tumors can also induce apoptosis in newly formed peripheral blood vessels, thereby ensuring the prevention of a relapse of cancer or cancer stem cell growth at an early stage[41]. The immunofluorescence-TUNEL technique has demonstrated that tumors in which HIF-1α expression is inhibited display increased DNA fragmentation[38]. Interestingly, although IL-8 does not exert a direct influence on tumor apoptosis, it plays a role in tumor apoptosis by controlling apoptosis in blood vessels[10]. Cultivated tumor cell lines with simultaneous inhibition of these two factors demonstrated increase in tumor apoptosis via the FACS-TUNEL technique. It was also confirmed that a vascular endothelial cell culture medium developed from a culture medium, in which apoptosis had been induced in tumor cells, promoted apoptosis in vascular endothelial cells without any stimulation (Fig 2)[23]. Apoptosis in tumors affects the surrounding tissues owing to the constant communication and transmission between cells. The blood vessels, which are essential for tumor growth, mutually communicate via various factors that are present in the vicinity of the tumor. Therefore, cancer treatment and anticancer drugs can induce apoptosis in tumors while simultaneously regulating the expression of an activation factor in vascular endothelial cells, a higher anti-tumor therapeutic efficacy can be achieved during treatment of tumors[42]. In addition, a more precise tumor treatment can be developed by eliminating the factors that support the growth

of malignant tumors, which relapse due to various reasons after tumor treatment.

Tumors can be treated using various methods and with several drugs; the efficacy of these treatments can confirmed via different experiments. Moreover, various studies have been conducted to develop potential anti-tumor treatments that work by regulating the microenvironment of the tumor or controlling various tumor growth factors along with the existing tumor treatments. Inhibition of the hypoxic mediator, HIF-1α, and the activation factor in V-E cells, IL-8, which is closely related to tumor development, can potentially be used to develop a treatment that can directly regulate tumor development as well as the microenvironment of tumors.

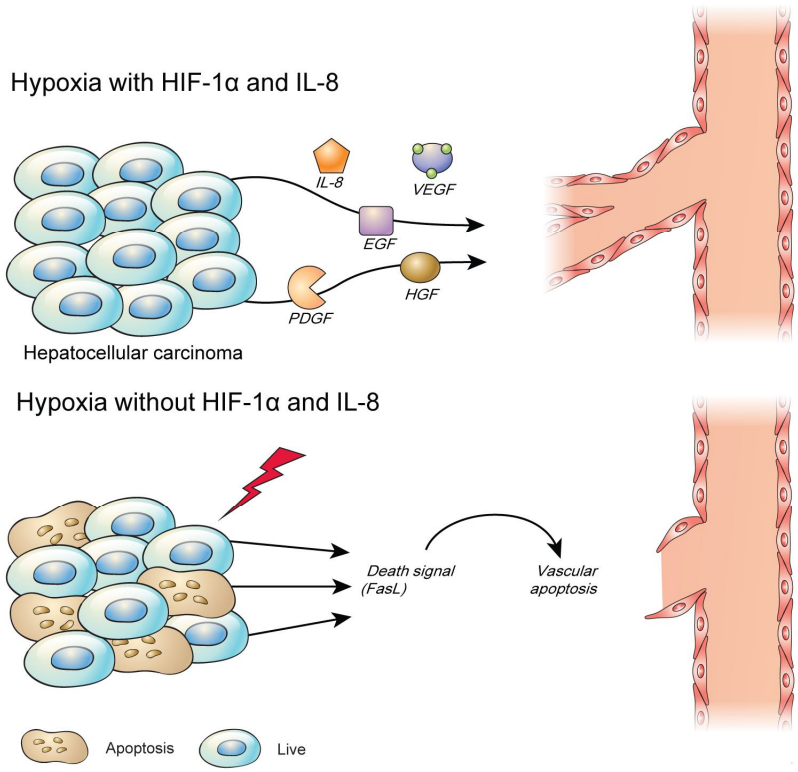


Figure 2. Regulation of hypoxic apoptosis in hepatocellular carcinoma. Apoptosis is an important mechanism for the development of organisms. Organisms survive and proliferate in the cyclic structure of cell creation and death. However, apoptosis is critical for inhibiting the growth of cancer cells. One of the significant survival mechanisms of cancer cells is the suppression or prevention of apoptosis. Adenovirus-mediated knockdown of HIF-1α and IL-8 induced apoptosis in HCC and triggered apoptosis of vascular endothelial cells.

II. MATERIALS AND METHODS

1. Viruses and shRNA

Nineteen base-pair shRNA sequences targeting HIF-1α and IL-8 genes (shHIF-1α and shIL-8, respectively) were prepared as previously described (14). The adenovirus E1 site[15], which is responsible for the proliferation of the adenovirus, was removed by BamHI restriction to produce replication-incompetent control virus Ad-ΔE1. Then, an RNA loop sequence forming a small hairpin structure specific to HIF-1α or IL-8 gene was inserted in its place, producing viruses Ad-shHIF-1α and Ad-shIL-8, respectively (Fig 3).

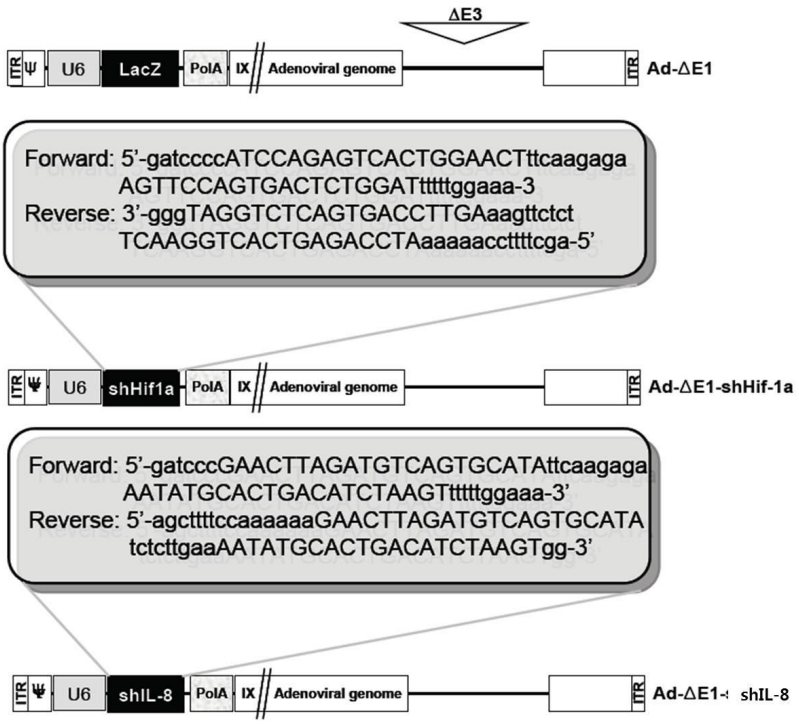


Figure 3 Adenovirus mediated shRNA construction. RNA loop sequence (19 base-pairs) forming a small hairpin structure specific to HIF-1α or IL-8 gene was inserted in E1 site, producing viruses Adenovirus mediated shHIF-1α (Ad-shHIF-1α) and shIL-8 (Ad-shIL-8).

2. small interfering RNA(siRNA)

Two knockdown systems were constructed by Adenovirus mediated shRNA[6] and piLentiviral vector mediated siRNA. Twenty nine nucleotide siRNA sequences targeting the HIF-1α and IL-8 genes (siHIF-1α and siIL-8, respectively) were prepared as previously described[52,61]. The piLenti vector incerted an RNA loop sequence forming a small hairpin structure specific to the HIF-1α (5'-GCTGGTGATTTGGATATTGAAGATGACAT-3'or
IL-8 (5'-TCTCTTGGCAGCCTTCCTGATTTCTGCAG-3') gene (abm, Richmond, BC, Canada), producing small interference HIF-1α and IL-8, respectively

3. Cell cultivation

Huh-7 (KCLB60104, Korean Cell line Bank), Hep3B[11], and HepG2 (KCLB88065, Korean Cell line Bank) cells were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Welgene, Daegu, Korea), 4.5 g/L glucose, 2% L-glutamine, and 1% penicillin/streptomycin

4. Adenovirus infection and transfection

Culture medium [DMEM containing 10% fetal bovine serum (FBS)] was replaced with a fresh medium containing 5% FBS before adenovirus infection. HCC cell lines (HepG2, Hep3B, Huh7) were then infected with Ad-shHIF-1α, Ad-shIL-8, or both Ad-shHIF-1α and Ad-shIL-8. Control cells were infected with a null virus (Ad-ΔE) or an adenovirus expressing shRNA for green fluorescent protein (Ad-shGFP). The infected cells were incubated for 24 h under normoxic conditions (21% O_2), followed by another 24 h incubation with a fresh medium under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2).

Culture medium was replaced with fresh medium containing 5% fetal bovine serum before transfection. Cells were then transfected with piLenti-siHIF-1α,

piLenti-siIL-8, or both. Control cells were transfected with an piLenti vector for green fluorescent protein (piLenti-siGFP). The transfected cells were incubated for 24 h under normoxic conditions (21% O_2), followed by another 24-h incubation with fresh medium under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2).

5. Cell growth assay

Cell growth was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. First, the HCC cells were seeded in a 96-well plate at 5 x 10^3 cells/well, incubated at 37°C for 24 h, transferred to serum-free medium, and infected with recombinant adenovirus as described above. After 24 h incubation under 21% O2, the cells were transferred to a culture medium containing 10% FBS, and hypoxia was induced by growth under 1% O_2. Finally, MTT was added to the cells, and the plates were incubated at 37°C for 3–4 hours for efficient cell dyeing. The culture supernatant was removed, treated with dimethylsulfoxide for 5 min at room temperature, and analyzed for its absorbance at 595 nm using a spectrophotometer.

6. Real-time quantitative PCR

Total RNA was extracted from HCC cells (Hep3B, Huh7) using Trizol reagent (Gibco-BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Precipitated RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water, and its amount and quality were assessed by measuring A260 and A280 on a NanoDrop (ND-100) spectrophotometer (Laboratory & Medical Supplies, Tokyo, Japan). One microgram of RNA was used as a template for cDNA synthesis using reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The synthesized cDNA

was then quantified using real-time quantitative PCR.

Table 1. Primer sequence

Genes	Forward sequence (5'~3')	Reverse sequence (5'~3')
HIF-1α	GTC TCA CGA GGG GTT TCC CG	GCC GAG ATC TGG CTG CAT CT
HIF-1β	CAG AGG GCT ATT AAG CGA CG	ATA CAG TGT GCT CCC GAA CC
IL-8	CAC CTC AAG AAC ATC CAG AGC	CAA GCA GAA CTG AAC TAC CAT
VEGF	TGC ACC CAT GGC AGA AGG AG	TGT GCT GGC CTT GGT GAG GT
EGF	CTT GTC ATG CTG CTC CTC CTG	TGC GAC TCC TCA CAT CTC TGC
HGF	CTC ACA CCC GCT GGG AGT AC	TCC TTG ACC TTG GAT GCA TTC
FGF2	CTG GCT ATG AAG GAA GAT GGA	TGC CCA GTT CGT TTC AGT G
MMP-2	TCT CCT GAC ATT GAC CTT GGC	CAA GGT GCT GGC TGA GTA GAT
MMP-9	TTG ACA GCG ACA AGA AGT GG	GCC ATT CAC GTC GTC CTT AT
Caspase-3	CAA ACT TTT TCA GAG GGG ATC	GCA TAC TGT TTC AGC ATG GCA
Bcl-xl	GAT CCC CAT GGC AGC AGT AAA	CCC CAT CCC GGA AGA GTT CAT
β-actin	GTG GGA GTG GGT GGA GGC	TCA ACT GGT CTC AAG TCA GTG

7. Immuno-blot

Total proteins were collected from Ad-shHIF-1- or Ad-shIL-8-infected HCC cells after they were incubated under hypoxic conditions for 24 h. The extracted proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride membrane (GE Healthcare, Amersham, Buckinghamshire, UK), and probed with mouse monoclonal antibodies specific for each protein of interest antibodies for HIF-1α (Bethyl, Montgomery, TX, USA), antidody for caspase family and bcl family (Cell Signaling, Danver, MA, USA), antidody for angiopoietin1, MMP-2 and MMP-9 (Abcam, Cambridge, MA, USA). The blots were developed using the ECL technique (PerkinElmer, Boston, MA, USA) according to the manufacturer's instructions, and the level of expression of each

protein was quantified and compared. For the detection of secreted proteins (EGF, HGF) and intracellular NO, an enzyme-linked immunosorbent assay and NO detection (ELISA; R&D Systems, Mineapolis, MN, USA) were performed according to the manufacturer's instructions.

Table 2. List of antibody for western blot

Antibody	Source	Dilution	Company
HIF-1α	Rabbit	1/1000	Bethyl
HIF-1β	Mouse	1/1000	Abcam
VEGF	Rabbit	1/1000	Millipore
MMP-2	Mouse	1/1000	Abcam
MMP-9	Mouse	1/1000	Abcam
Caspase-3	Mouse	1/1000	Cell signaling
Caspase-8	Mouse	1/1000	Cell signaling
Caspase-9	Mouse	1/1000	Cell signaling
Bcl-xL	Rabbit	1/1000	Cell signaling
Bcl-2	Mouse	1/1000	Cell signaling
Angiopoietin-1	Rabbit	1/1000	Abcam
β-actin	Mouse	1/5000	Sigma

8. Immunoprecipitation

Total cell lysate was extracted from HCC cells using RIPA cell lysis buffer (Cell signaling) according to the manufacturer's instructions. HIF-1α was bounded with mouse anti-HIF-1α (Cell signaling) using IgG magnetic beads (Novex, Oslo, Norway), according to the manufacturer's instructions. The dimerized HIF-1β proteins were detected according to western blot, and blotted with the other target-mouse monoclonal antibodies-HIF-1α (Bethyl) specific for the proteins of interest. The blots were developed using the enhanced chemiluminescence technique (PerkinElmer) according to the manufacturer's instructions, and the level of expression of each protein was quantified and

compared

9. Angiogenesis Assays

Angiogenesis was investigated *in vitro* by examining invasiveness, tube formation, and vessel sprouting, using HUVECs grown in conditioned medium collected from the cells of HIF-1α or IL-8 knockdown. (i) HUVEC invasiveness was assessed in vitro using a transwell chamber (Corning Costar, Cambridge, MA, USA). Each transwell chamber was plated with 7 x 10^4 HUVECS, and the invading cells were stained with hematoxylin and eosin. The total number of invaded cells on the lower side of the filter was counted under the microscope (Olympus America, Melville, NY, USA) at 40x magnification. (ii) Tube formation by HUVECs was measured in Matrigel (BD Biosciences, San Jose, CA, USA). A 96-well plate (BD Falcon, Bedford, MA, USA) was coated with Matrigel (10 mg/ml), plated with HUVECs (7 x 10^4/well), and cultivated for 5 h. Tube formation was then observed under the light microscope. (iii) Vessel sprouting was measured using an ex-vivo aortic ring-sprouting assay in which microvessels were sprouted from aortas harvested from 6-week-old Sprague–Dawley rats as previously described[28]. After removal of the surrounding fibro-adipose tissues, the aortas were rinsed with Hank's balanced salt solution buffer (Gibco) and cut into rings. The rings were then placed in the wells of 48-well plates coated with Matrigel. After 5–6 days, each ring was scored from 0 (least positive) to 5 (most positive), according to the degree of vessel sprouting observed.

10. Wound healing assay

The mobility of cells was assessed by scratch and wound healing assay. Each experimental result was observed by an optical microscope.

11. Tumor xenograft model

Hep3B human tumor xenografts were established in 6- to 8-week-old male, athymic, nu/nu mice (Charles River Japan, Inc., Yokohama, Japan) by subcutaneous implantation of 1 x 10^7 Hep3B cells in the abdominal region (10 mice/group). When tumor volumes reached to approximately 100 mm^3 (calculated by volume = 0.523 LW^2), the animals were sorted into groups of similar mean tumor volumes, and adenovirus or phosphate-buffered saline (PBS) was injected into the tumors.

12. Immunohistochemical analysis

Tumor tissues were fixed in formalin and formalin-free IHC zinc fixative (BD Biosciences Pharmingen, San Diego, CA, USA), embedded in paraffin (Wax-it, Vancouver, BC, Canada), and cut into 4-μm sections. Representative sections were stained with hematoxylin and eosin and examined by light microscopy. For immunohistochemical analysis, slides were deparaffinized in xylene and then hydrated with graded ethanols. The endogenous peroxidase in the sections was blocked with 3% hydrogen peroxide. Sections were incubated with a serum-free blocking agent (Dako, Carpinteria, CA, USA), incubated at 4°C overnight with primary antibody [HIF-1α (Norvous, Littleton, CO, USA), IL-8 (R&D system), CD31 and CD34 (BD pharmingen, Franklin Lakes, NJ, USA), VE cadherin (Abcam), VEGFR2 (Cell Signaling)], and rinsed in PBS-T (0.05% Triton X-100 in PBS). They were then incubated with a secondary antibody (1:500), washed with PBS, and incubated with streptavidin–horseradish peroxidase (Dako, Carpinteria, CA, USA). All slides were counterstained with Meyer's hematoxylin and observed under the microscope (Olympus America) at 40x or 100x magnifications.

13. Apoptosis assay

Cells were stained with FITC-labeled annexin V and propidium iodide (PI), and tumor cell death was assessed by terminal deoxynucleotidyl transferase

dUTP nick end labeling (TUNEL, Promega) assay and flow cytometry

14. Immunofluorescence and TUNEL-fluorescence

Tumor tissues were fixed in formalin and formalin-free IHC zinc fixative (BD Biosciences Pharmingen, San Diego, CA, USA), embedded in paraffin (Wax-it), and cut into 4-μm-thick sections. Representative sections were stained with hematoxylin and eosin and examined by light microscopy. For immunofluorescence analysis, slides were deparaffinized in xylene and then hydrated with graded ethanols. antigen was retrieved in a citrate buffer solution. Sections were incubated with 1% bovine serum albumin (BSA) buffer (Perkinamer, Carpinteria, CA, USA), incubated at 4°C overnight with primary antibody VEGFR2 (Cell Signaling), and rinsed in PBS-T (0.05% Triton X-100 in PBS). They were then incubated with a secondary antibody (1:500), washed with PBS, and incubated with streptavidin–horseradish peroxidase (Dako). TUNEL-fluorescence was detected using a TUNEL-fluorescence kit (Promega). Tissues were embedded in paraffin and stained with FITC-labeled annexin V. All slides were observed under a fluorescence microscope (Olympus America, Melville, NY, USA, and Carl Zeiss) at 40× magnification.

15. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) or frequency (%). Statistical analysis was performed using the independent t-test with SPSS software version 12.0 (SPSS, Inc., Chicago, IL, USA). Differences between control and experimental groups were considered statistically significant at $P < 0.05$.

III. RESULTS

PART I

Inhibition of tumor angiogenesis and growth by small hairpin HIF-1α and IL-8 in hepatocellular carcinoma

1. Inhibition of tumor cell growth *in vitro* by adenovirus-mediated knockdowns of HIF-1α and IL-8

The effect of Ad-shHIF-1α and Ad-shIL-8 infection on HCC cell proliferation was examined by infecting Hep3B cells with Ad-shHIF-1α and Ad-shIL-8 simultaneously at various multiplicities of infection (MOIs). Adenoviruses expressing shHIF-1α and shIL-8 did not replicate in HCC cells in vitro but effectively inhibited the expression of HIF-1α and IL-8. Seventy two hours after infection, the cells infected with Ad-shHIF-1α and Ad-shIL-8 at higher MOIs inhibited cell growth more efficiently than the control cells infected with Ad-shGFP. At MOIs of 60, 100, and 120, proliferation was inhibited dose dependent, respectively (each P-value < 0.05) (data not shown). Under both normoxic and hypoxic conditions, the inhibitory effect on the cell growth was more substantial in cells infected with both Ad-shHIF-1 α and Ad-shIL-8 than that in the control cells.

The relationship between cell growth inhibition and MOI was examined to address whether the observed growth inhibition was the direct effect of suppression of the target genes or the effect of virus infection. When the growth rate of each HCC cell line (HepG2, Huh7, or Hep3B) was examined for its dependence on MOI, no significant effect was observed for the cells infected with Ad-shIL-8 or control virus. However, cell growth was inhibited by high MOIs of Ad-shHIF-1α infection (Fig 4A).

Differences of cell growth between the virus-infected knockdown groups and control groups were observed first on day 2 after infection. On day 4, growth of the Hep3B cells which were simultaneously infected with Ad-shHIF-1α and Ad-shIL-8 under hypoxia was inhibited by 67% relative to the control group under hypoxia, whereas their growth under normoxia was inhibited by approximately 50% relative to the control group (Fig 4B).

To address whether EGF and/or HGF play roles in the growth of HIF-1α - and IL-8-co-silenced hepatoma cells, the cells were exogenously treated with EGF and/or HGF and cell growth rate was measured by MTT assay. As shown in Figure 4C, under hypoxic condition, EGF and/or HGF exerted positive effects on the cell growth of HIF-1α - and IL-8-co-silenced hepatoma cells, however this effect was not evident under normoxic condition.

Thus, adenovirus-mediated shHIF-1α expression inhibited growth of all the HCC cell lines under hypoxic conditions, whereas adenovirus-mediated shIL-8 expression had no direct effect on growth of any of the HCC cell lines.

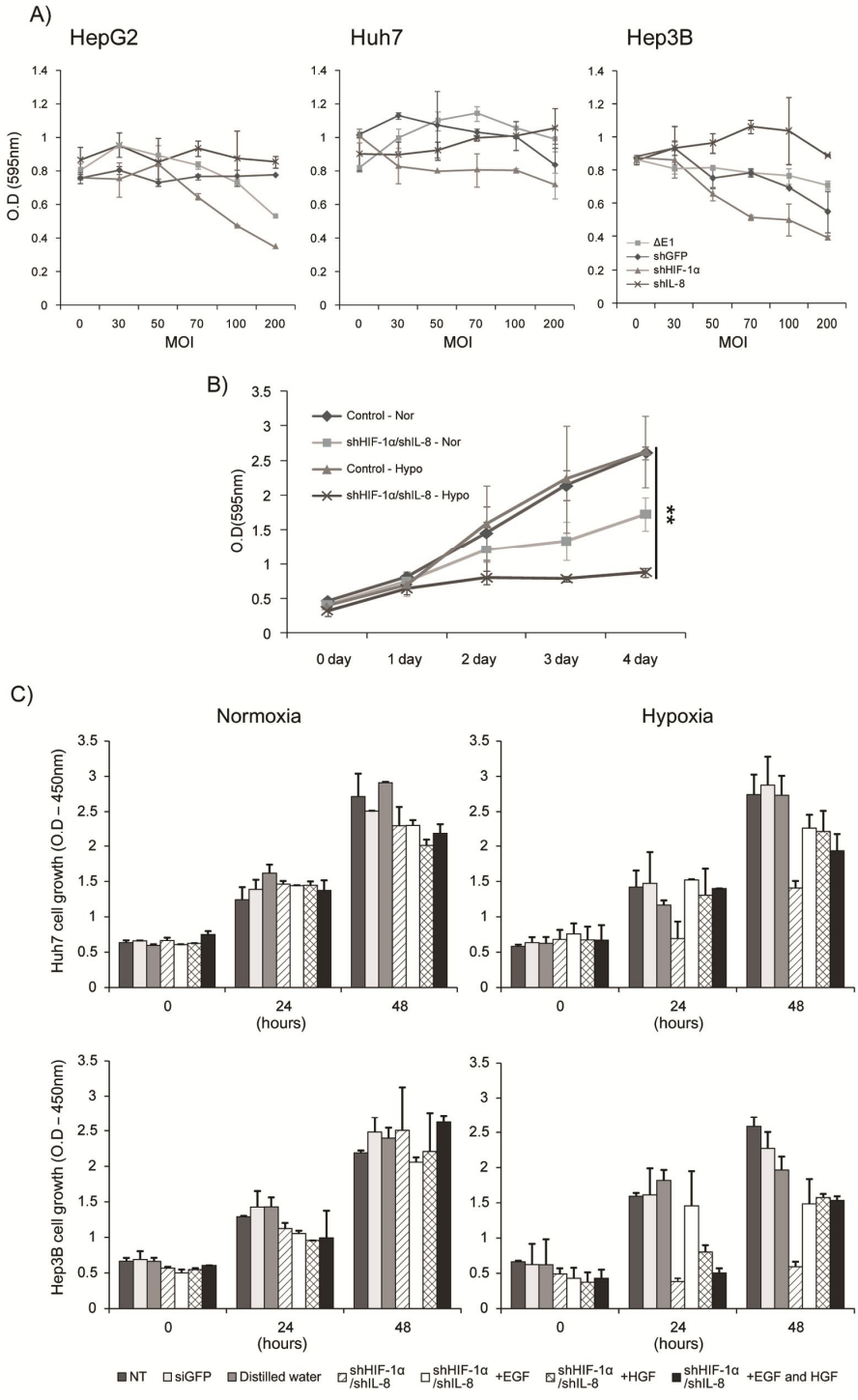

A)
HepG2
Huh7
Hep3B
O.D (595nm)
MOI
ΔE1
shGFP
shHIF-1α
shIL-8
B)
O.D(595nm)
Control - Nor
shHIF-1α/shIL-8 - Nor
Control - Hypo
shHIF-1α/shIL-8 - Hypo
**
0 day
1 day
2 day
3 day
4 day
C)
Normoxia
Hypoxia
Huh7 cell growth (O.D – 450nm)
Hep3B cell growth (O.D – 450nm)
(hours)
NT
siGFP
Distilled water
shHIF-1α /shIL-8
shHIF-1α /shIL-8 +EGF
shHIF-1α /shIL-8 +HGF
shHIF-1α /shIL-8 +EGF and HGF

Figure 4. Inhibition of cell growth *in vitro* by Ad-shHIF-1α and Ad-shIL-8. (A) Cytotoxicity of Ad-shHIF-1 α and Ad-shIL-8 in HCC cell lines measured by MTT assay. Under hypoxic conditions, cells infected with Ad-shHIF-1α showed a dose-dependent toxicity, whereas this finding was not evident in cells infected with Ad-shIL-8. (B) Silencing of HIF-1α and IL-8 in tumor cells under normoxic (Nor) or hypoxic (Hypo) condition demonstrated that silencing of HIF-1α and IL-8 inhibits cell growth under hypoxic condition. [*P < 0.05 vs. control (Ad-ΔE1)]. (C) Treatment of EGF and HGF did not respond under normoxia. Silencing of HIF-1α and IL-8 inducing cell growth inhibition was retrieved by treatment of EGF and/or HGF, partly [*P < 0.05 vs. Silencing of HIF-1α and IL-8 group].

2. Downregulation of angiogenic factors in Ad-shIL-8- and Ad-shHIF-1α-infected Hep3B cells.

Quantitative real-time PCR measurements of HIF-1α and IL-8 mRNA levels showed that their abundances were approximately 57% and 73% lower in the Ad-shHIF-1α - and Ad-shIL-8-infected cells, respectively compared to those of non-infected control group (Fig 5A), confirming that infection with Ad-shHIF-1α or Ad-shIL-8 resulted in knockdown of HIF-1α or IL-8 expression.

To investigate the effect of HIF-1α and IL-8 knockdown on the expression of other factors involved in angiogenesis, I examined the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) (Fig 5B). Compared to the negative control (Ad-ΔE1-infected cells), the gene expression of VEGF and MMP-2 was significantly diminished by co-silencing of HIF-1α and IL-8. This result implies that the expression of VEGF and MMP-2 is regulated by HIF-1α and IL-8.

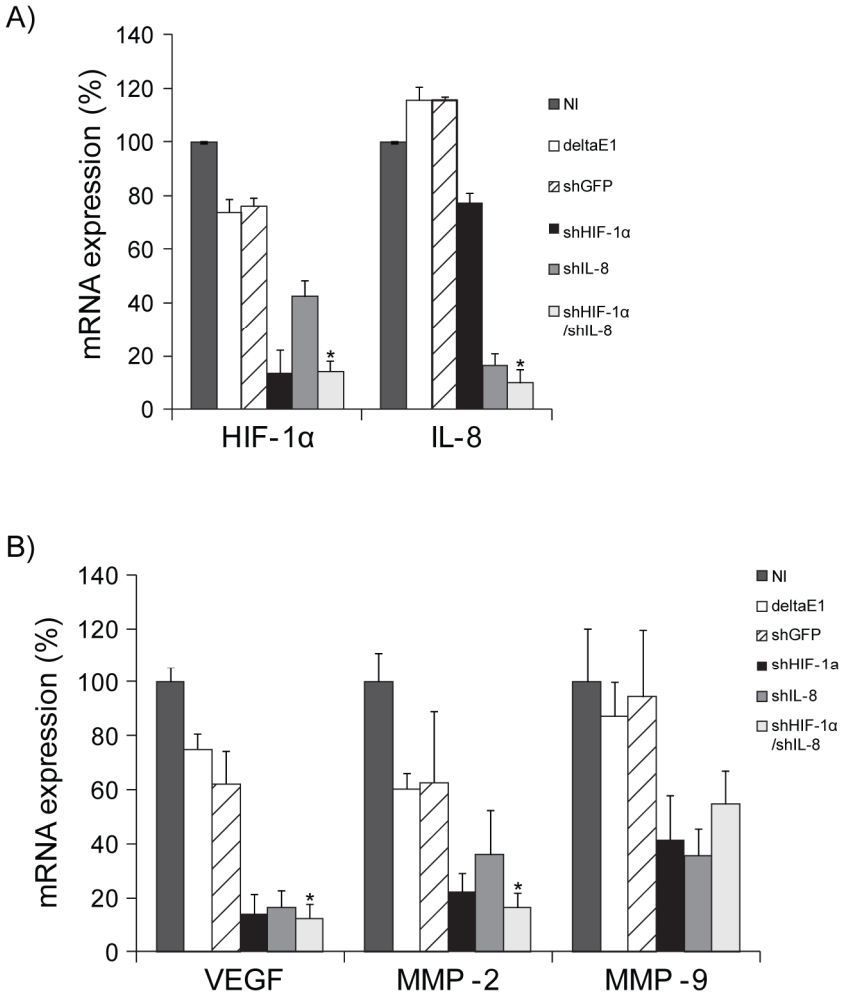


Figure 5. mRNA expression of Ad-shHIF-1α and shIL8 infected Hep3B cells. (A) Ad-shHIF-1α and Ad-shIL-8 infection reduced the abundance of HIF-1α mRNA and IL-8 mRNA, respectively. (B) HIF-1α and IL-8 knockdown decreased the expression of VEGF, MMP-2, and MMP-9 mRNAs under hypoxic conditions [*$P < 0.05$ vs. control (Ad-shGFP infection)]. Target mRNA expression was normalized to β-actin mRNA expression.

3. The expression of HIF-1α was compared by immunoblot.

Quantification of HIF-1α showed that infection with Ad-shHIF-1α in Hep3B cells inhibited HIF-1α expression in an MOI-dependent manner (Fig 6A) by approximately 34% and 58% at MOIs of 30 and 60, respectively. Quantification of IL-8 by ELISA showed that IL-8 secretion by Ad-shIL-8-infected cells was approximately 70% lower than that by the control cells ($P < 0.05$) (Fig 6B).

Immunoblot of MMP-2 and MMP-9 showed that the expression level of these proteins was approximately 40–50% lower with Ad-shHIF-1α and Ad-shIL-8-infected cells than those of the control cells (Figure. 6C), which was consistent with the ELISA result of MMP-2 (Fig 6D). Next, I evaluated the effect of HIF-1α and/or IL-8 silencing on the expression of the key molecules involved in angiogenesis and vascular endothelial stabilization, namely, VEGF, angiopoietin1 and iNO (Fig 6E, F and G). Compared to the controls, the expression of VEGF was significantly decreased by HIF-1α and/or IL-8. Interestingly, the expression of angiopoietin1 and iNO was substantially reduced by silencing of HIF-1α alone or together with IL-8, whereas silencing of IL-8 alone did not affect their expression. The expression of HGF and EGF was also reduced by HIF-1α and both HIF-1α- and IL-8-knockdown under hypoxia (Fig 6G). The abundance of EGF was approximately 10–20% less than that of the control groups and the abundance of HGF was approximately 10% less than those of the control groups. Under normoxia, there was no discrepancy between the control and knockdown groups concerning the level of EGF and HGF (data not shown). Taken together, these results suggest that HIF-1α and IL-8 regulate expression of angiogenic and growth factors under hypoxic conditions.

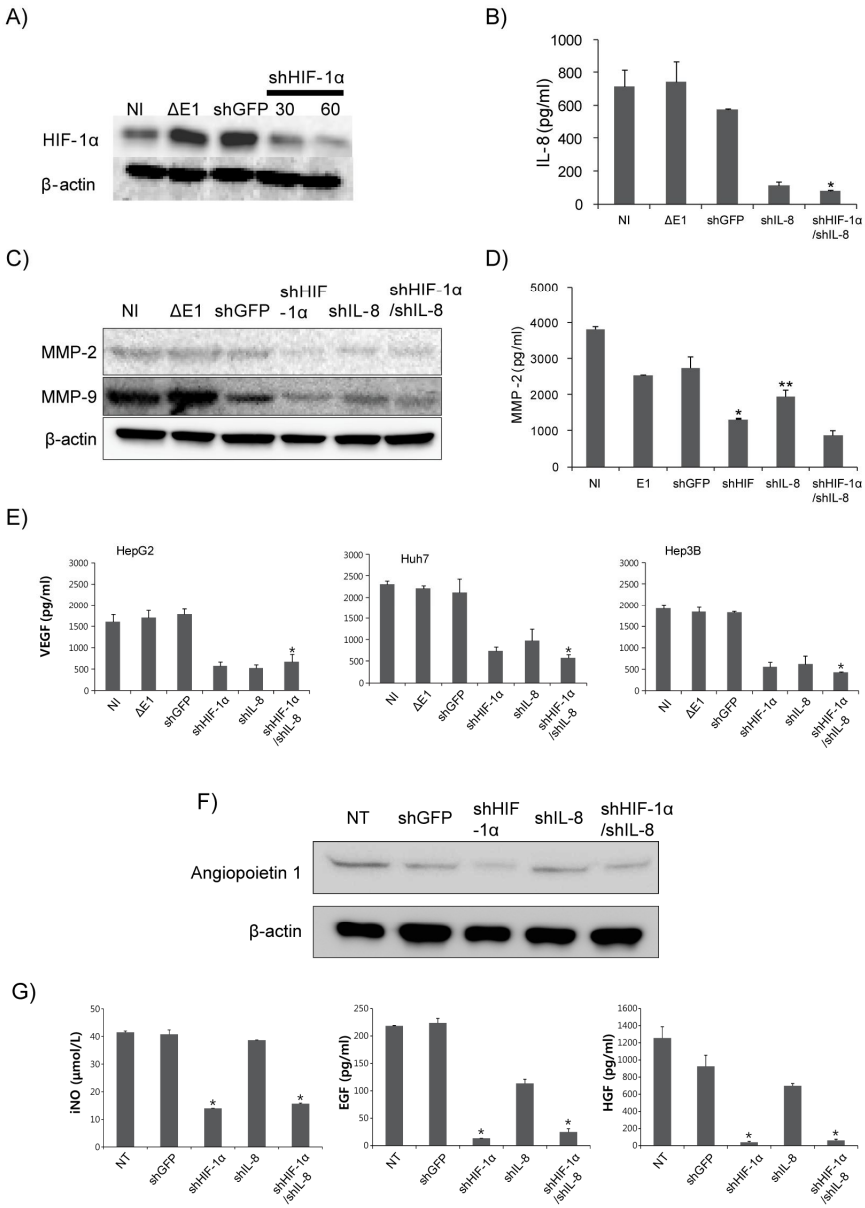


Figure 6. Effect of HIF-1α and IL-8 knockdown on the expression of angiogenic factors in Hep3B cells. (A) Immunoblot of HIF-1α as a loading

control. The abundance of HIF-1α protein was reduced in an MOI-dependent manner under hypoxic conditions. (B) ELISA of IL-8 secreted from the infected cells under hypoxic conditions. (C) Immunoblot of MMP-2 and MMP-9 showing reduced levels of the proteins in Ad-shHIF-1α- and Ad-shIL-8-infected cells under hypoxic conditions. (D) ELISA result of MMP-2 confirming decreased levels of MMP-2 in cells simultaneously infected with Ad-shHIF-1α and Ad-shIL-8 [*$P < 0.05$ vs. control (Ad-shGFP infection)]. (E) ELISA of VEGF showing decreased levels of VEGF in cells infected with Ad-shHIF-1α or Ad-shIL-8 under hypoxic conditions. (F) Decreased expression of angiopoietin1 by HIF-1α knockdown under hypoxic conditions. (G) Decreased levels of iNO, EGF and HGF in Ad-shHIF-1α- and Ad-shIL-8-infected cells under hypoxic conditions.

4. Inhibition of HUVEC angiogenesis by conditioned medium from HIF-1α - and IL-8-knockdowns in Hep3B cells

The effect of HIF-1α and IL-8 suppression on cellular invasion, tube formation, and aortic microvessel formation was investigated using conditioned media collected from adenovirus-infected and non-infected Hep3B cells. In cellular invasion assay, hematoxylin and eosin staining of HUVECs treated with conditioned media from non-infected and Ad-shGFP-infected cells showed that most of the cells panetrated the membrane and were distributed in the gelatin area. Conditioned media from the cells of HIF-1α or IL-8 knockdown partially inhibited cellular invasion, and the media from the cells infected by both Ad-shHIF-1α and Ad-shIL-8 inhibited cellular invasion by approximately 70% (Fig 7A).

In tube formation assay, the media collected from the non-infected and Ad-shGFP-infected cells generated thick and clear tubes. However, the media from the cells infected by Ad-shHIF-1α or Ad-shIL-8 generated slightly thinner and disconnected tubes. Only a few disconnected tube segments were observed by treatment with the media obtained from the cells which were infected with both Ad-shHIF-1α and Ad-shIL-8 (Fig 7B).

In aortic sprouting assay, the media collected from the non-infected and Ad-shGFP-infected cells generated microvessels that branched outward from the aorta in multiple directions. The media from the cells of HIF-1α or IL-8 knockdown generated fewer and smaller microvessels. The media from the cells infected with both Ad-shHIF-1α and Ad-shIL-8 completely blocked microvessel sprouting (Fig 7C).

Collectively, these results demonstrated inhibition of angiogenesis by conditioned media of HIF-1α or IL-8 knockdown in HCC cells and the additive manner of such inhibition by knockdown of both HIF-1α and IL-8.

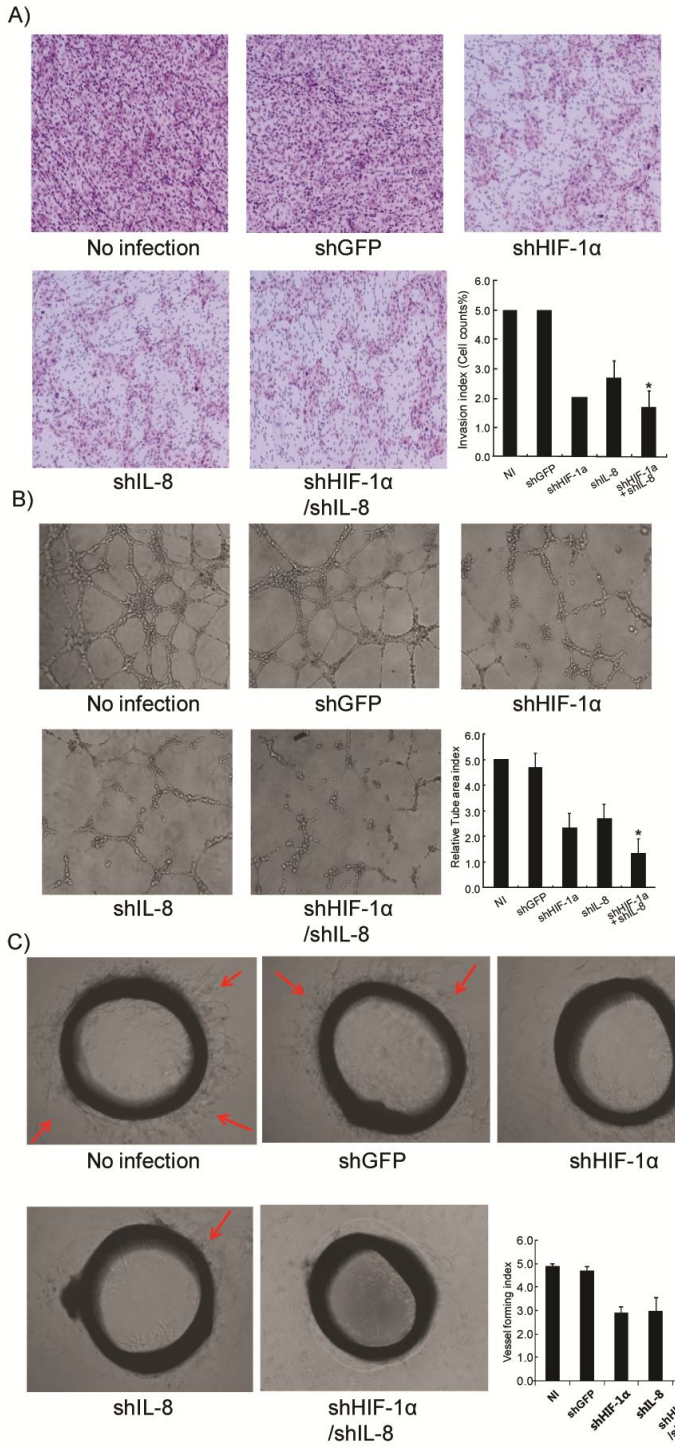

A)
No infection
shGFP
shHIF-1α
shIL-8
shHIF-1α
/shIL-8
Invasion index (Cell counts%)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
NI
shGFP
shHIF-1a
shIL-8
shHIF-1a
+shIL-8
B)
No infection
shGFP
shHIF-1α
shIL-8
shHIF-1α
/shIL-8
Relative Tube area index
C)
No infection
shGFP
shHIF-1α
shIL-8
shHIF-1α
/shIL-8
Vessel forming index
NI
shGFP
shHIF-1α
shIL-8
shHIF-1α
/shIL-8

Figure 7. Effects of conditioned media from HIF-1α- and IL-8-knockdown on HUVEC angiogenesis. (A) Media collected from non-infected or Ad-shGFP-infected Hep3B cells led to invasion by HUVECs. However, media from Hep3B cells infected with Ad-shHIF-1α or Ad-shIL-8 inhibited HUVEC invasion. (B) Media from non-infected or Ad-shGFP-infected Hep3B cells led to the formation of an extensive HUVEC network of organized, elongated, tube-like structures resembling capillaries. However, media from the cells infected with both Ad-shHIF-1α and Ad-shIL-8 inhibited tube formation. Only incomplete network of capillary-like structures formed. (C) Media from non-infected or Ad-shGFP-infected Hep3B cells enhanced growth of sprouting aortic microvessels. However, no microvessel sprouting was observed for the media collected from the cells infected with both Ad-shHIF-1α and Ad-shIL-8.

5. Anti-tumor effects of HIF-1α and IL-8 knockdowns in subcutaneous Hep3B tumors *in vivo*

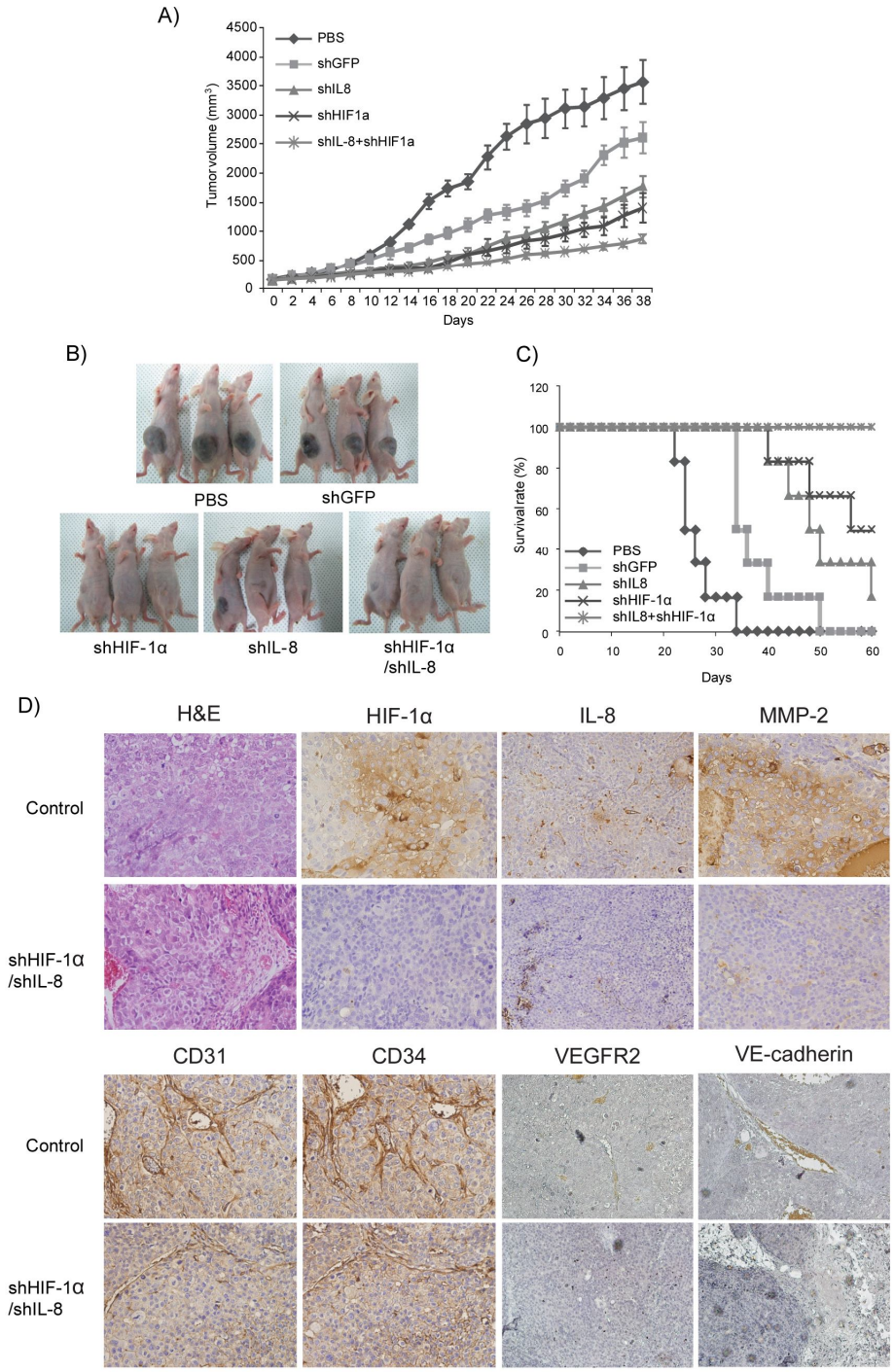
To investigate anti-tumor effect of shHIF-1α and shIL-8 expression *in vivo*, i used a subcutaneous Hep3B tumor xenograft model. In this model, Hep3B implantation-induced tumors in mice infected with Ad-shHIF-1α or Ad-shIL-8 were smaller than those of control mice treated with Ad-shGFP only. Tumor volume of the control groups increased up to 3,000 ~ 3,500 mm^3 over 38 days and those of single knockdown groups (Ad-shHIF-1α or Ad-shIL-8 infection) increased up to 1,500 ~ 2,000 mm^3. Additive effect of knockdown of both HIF-1α and IL-8 was shown by much smaller tumor volume of approximately 500 ~ 700 mm^3 (Fig 8A).

Expression of shHIF-1α in the induced tumors had a marked effect on tumor phenotype. Tumors in the control groups and the Ad-shIL-8-infected group were dark red and firm, whereas those from the Ad-shHIF-1α-infected and the group infected with both Ad-shHIF-1α and Ad-shIL-8 were white and soft. In addition, tumors from the group of infection with both viruses shrank to the point of being barely visible to the naked eye (Figure 8B). The survival rate of mice bearing Hep3B tumors was higher for those treated with Ad-shIL-8 or Ad-shHIF-1α than that for control mice treated with PBS only and was highest for the mice infected with both Ad-shIL-8 and Ad-shHIF-1α (Fig 8C). All mice survived even after 60 days for the group of infection with both Ad-shHIF-1α and Ad-shIL-8, thus demonstrating more efficient anti-tumor effect than the control or single knockdown groups.

6. Anti-angiogenic effects of HIF-1α and IL-8 knockdown on subcutaneous Hep3B tumors

In tumors from the control group, expression of both HIF-1α and IL-8 was

easily detected by immunohistochemical assay (Fig 8D). In contrast, expression of both HIF-1α and IL-8 was reduced in tumors from the mice infected with both Ad-shHIF-1α and Ad-shIL-8. The expression of MMP-2 (invasion marker), CD31 and CD34 (angiogenesis markers), and VE-cadherin and VEGF-R2 (vascular endothelial markers) was also reduced in the mice infected with both Ad-shHIF-1α and Ad-shIL-8 compared to those of the control mice. These results show that suppression of HIF-1α and IL-8 effectively inhibits expression of the angiogenesis-related proteins.


A)
Tumorvolume (mm3)
Days
PBS
shGFP
shIL8
shHIF1a
shIL-8+shHIF1a
B)
PBS
shGFP
shHIF-1α
shIL-8
shHIF-1α /shIL-8
C)
Survival rate (%)
Days
PBS
shGFP
shIL8
shHIF-1α
shIL8+shHIF-1α
D)
H&E
HIF-1α
IL-8
MMP-2
Control
shHIF-1α /shIL-8
CD31
CD34
VEGFR2
VE-cadherin
Control
shHIF-1α /shIL-8

Figrue 8. Effects of HIF-1α and IL-8 knockdown on tumor growth and expression of angiogenic factors in a tumor xenograft model. (A) Subcutaneous Hep3B tumors in mice infected with both Ad-shHIF-1α and Ad-shIL-8 grew more slowly than the tumors treated with Ad-shGFP. (B) Tumor sizes and phenotypes observed 6 weeks after adenovirus infection. (C) The survival rate was highest in the mice infected with both Ad-shHIF-1α and Ad-shIL-8. (D) Mice infected with both Ad-shHIF-1α and Ad-shIL-8 showed reduced HIF-1α and IL-8. They also showed reduced expression of angiogenesis markers, CD31 and CD34, and endothelial markers, VE-cadherin and VEGF-R2. Representative data of three times independent experiment are shown.

PART II

Silencing of hypoxia-inducible factor-1β induces anti-tumor effects in hepatoma cell lines under tumor hypoxia

1. Silencing of HIF-1α and HIF-1β suppresses tumor cell growth

After transfection of HCC cells with various concentrations of siHIF-1β, tumor cell growth was assessed by MTT assay. Forty-eight hours after transfection, tumor cell growth was significantly suppressed as compared to control, in a dose-dependent manner. The negative effect of HIF-1β silencing on tumor cell growth was more prominent under hypoxic conditions, especially when more than 100 nM of siHIF-1β or siHIF-1α was transfected to the tumor cells (Fig 9A, and Fig 10). Although the tumor cell growth was maintained at higher doses of siHIF-1β transfection under normoxic conditions, exposure to hypoxic environment resulted in significant suppression of tumor cell growth. Suppression of tumor cell growth under hypoxic conditions by HIF-1β-silencing was more pronounced by prolonged exposure to hypoxic environment. (Fig 9B). Of note, consistent with the previous findings, silencing of HIF-1α also displayed suppression of tumor cell growth under hypoxic conditions, which is probably mediated by inhibition of several other targets related to cell proliferation. These findings demonstrate that tumor cell growth is suppressed by silencing of HIF-1β under hypoxic conditions.

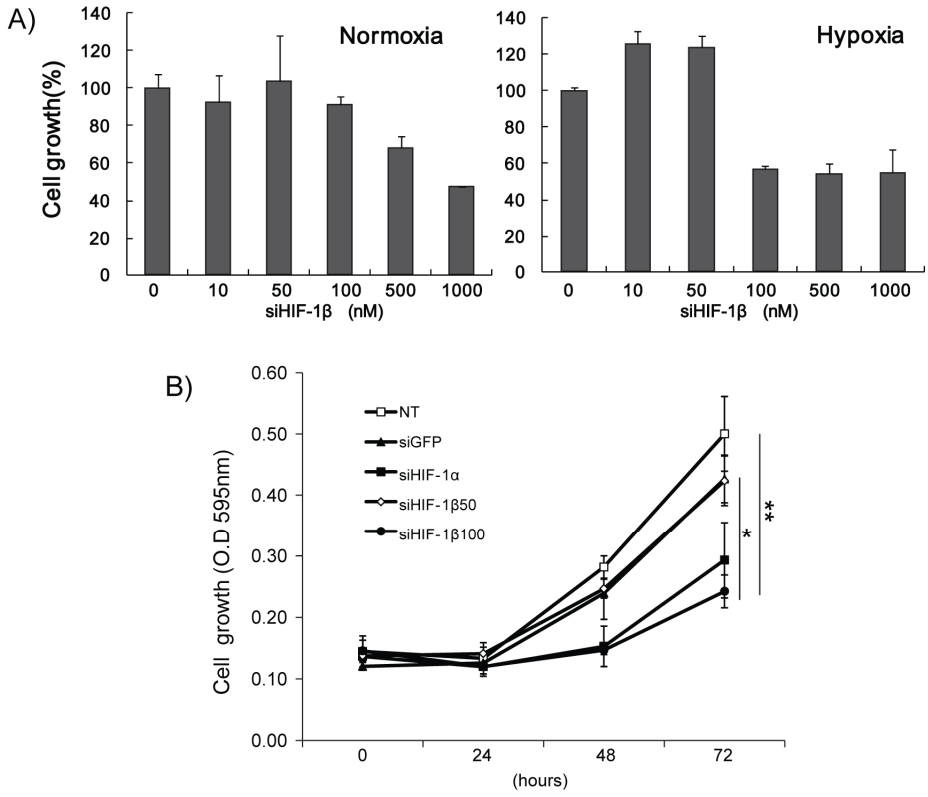


Figure 9. Suppression of tumor cell growth by silencing of hypoxia-inducible factors-1α and -1β. Huh-7 cells were transfected with small interfering RNAs against HIF-1α, HIF-1β, or green fluorescent protein as control (siHIF-1α, siHIF-1β, and siGFP, respectively), followed by exposure at hypoxic conditions (1% O2). (A) Tumor cell growth after silencing of HIF-1α or -1β was measured by MTT assay. Tumor cells were susceptible to growth retardation under hypoxic conditions when more than 100 nM of siHIF-1β was transfected. However, normoxic conditions (100 nM) did not show significant difference. (B) Transfection of siHIF-1α (100 nM) or siHIF-1β (100 nM) suppressed cell growth when cell were maintained at hypoxic conditions. As compared to control, the growth inhibition was more prominent with increase of siHIF-1β concentration. NT, non-target; siGFP, siRNA against green

fluorescent protein; siHIF1-α, siRNA against HIF-1α; siHIF-1β, siRNA against HIF-1β. *, $P < 0.05$; **, $P < 0.01$.

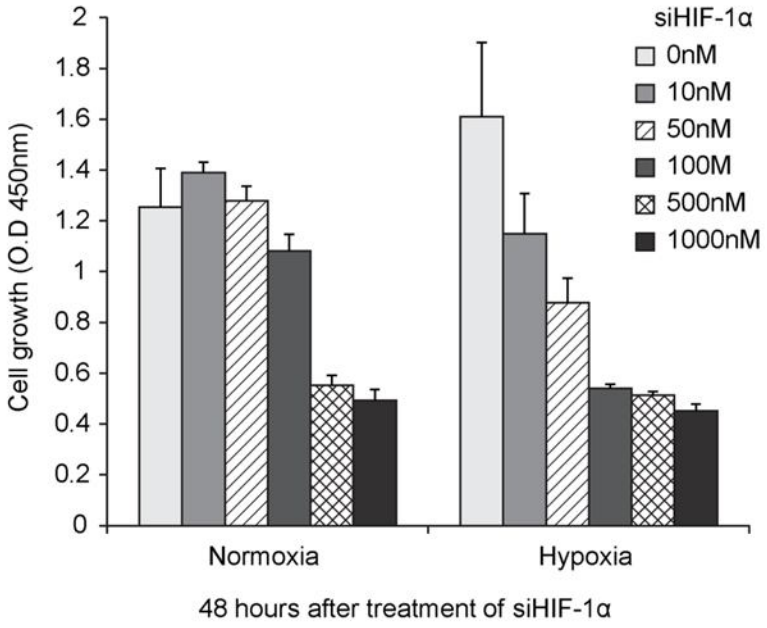


Figure 10. Suppression of tumor cell growth by knockdown of hypoxia-inducible factors-1α. Tumor cell growth after knockdown of HIF-1α was measured by MTT assay. Tumor cells were susceptible to growth inhibition under hypoxic conditions when more than 100 nM of siHIF-1α was transfected. However, normoxic conditions (100 nM) did not show significant difference.

2. Silencing of HIF-1β affects expression of tumor growth-related genes

Since tumor cell growth was inhibited by HIF-1β-silencing, i measured the mRNA levels of growth factors involved in tumor cell growth in HIF-1β-silenced tumor cells. Expression of HIF-1β was reduced by >60% in the group treated with siHIF-1β compared with that in the Control group, in a cell-density-dependent manner. Therefore, siHIF-1β inhibits expression of HIF-1β. HIF-1β knockdown did not affect HIF-1α expression. To determine the influence of HIF-1β knockdown on genes related to tumor growth, the mRNA expression levels of EGF, FGF2, and HGF were quantified by real-time quantitative PCR (Fig 11). Under hypoxic conditions, silencing of HIF-1β produced diminished expression of EGF and HGF by 52% and 36% (p-value < 0.05), respectively, compared to control. However, mRNA expression level of FGF2 expression was not affected by HIF-1β-silencing. Collectively, these data suggest that under hypoxic conditions, HIF-1β expression regulates the expression of tumor growth-related factors, namely EGF and HGF, but not FGF2.

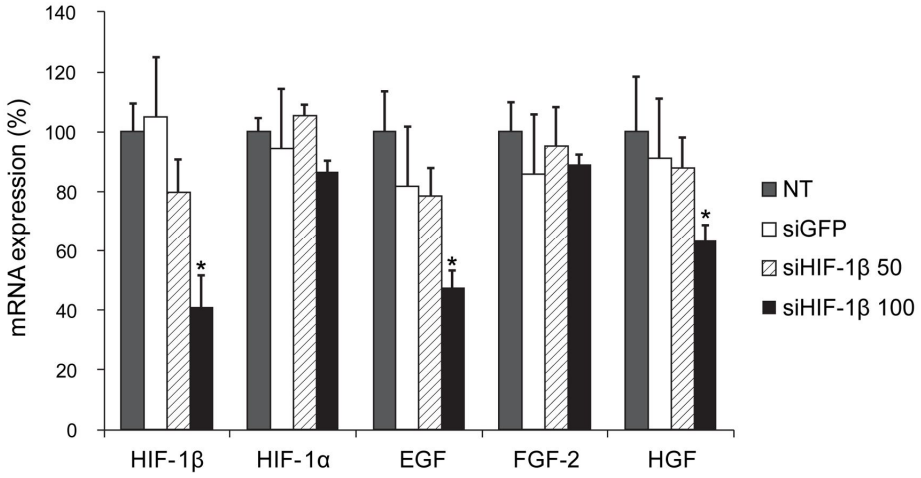


Figure 11. Silencing of HIF-1β affects expression of tumor growth-related genes. Under hypoxic conditions, silencing of HIF-1β was associated with diminished expression of several genes related to tumor growth, such as EGF and HGF, but not FGF2. NT, non-target; siGFP, siRNA against green fluorescent protein; siHIF1-α, siRNA against HIF-1α; siHIF-1β, siRNA against HIF-1β; EGF, epidermal growth factor; HGF, hepatocyte growth factor; FGF2, fibroblast growth factor 2. *, $P < 0.05$.

3. Silencing of HIF-1β affects protein expression and secretion of tumor growth-related genes

Based on the aforementioned mRNA data, protein expressions of tumor growth-related genes were analyzed using HIF-1β silenced Hep3B cells. Dose-dependent and selective inhibition of HIF-1β expression by siHIF-1β transfection was confirmed by immunoblot assays (Fig 12A). Interaction of HIF-1β and HIF-1α was confirmed by immunoprecipitation (Fig 13). IP results demonstrated that decreased protein expression levels of HIF-1β or HIF-1α in HIF-1α tagged group of HIF-1β silenced Hep3B cells. To determine the influence of HIF-1β inhibition on factors related to tumor cell growth, the levels of protein expression and secretion of EGF, HGF, VEGF, and FGF2 were analyzed (Fig 12B). ELISA results demonstrated that decreased protein expression levels of EGF, HGF, and VEGF in HIF-1β-silenced cells. Of note, the expression of these molecules was also decreased in HIF-1α-silenced cells. Notably, the expression of FGF2 was not affected by silencing of HIF-1α or HIF-1β. Collectively, these results demonstrate that under hypoxic conditions, HIF-1β expression regulates the expression of various tumor growth-related factors, namely EGF, HGF, and VEGF, but not FGF2.

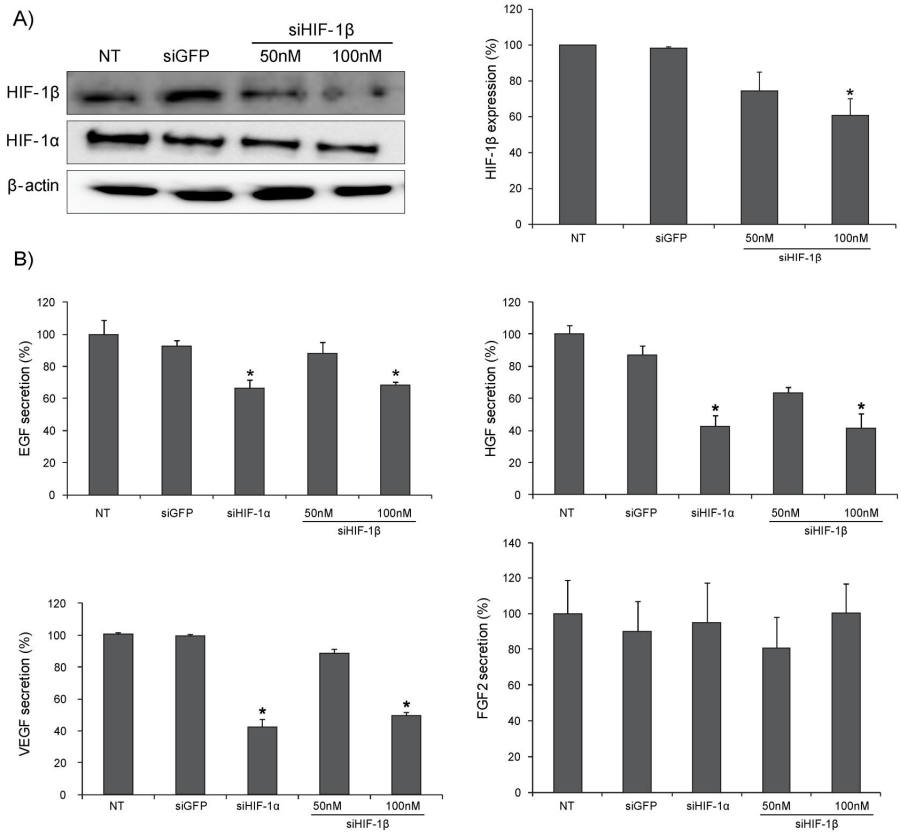


Figure 12. Silencing of HIF-1β affects protein expression and secretion of tumor growth-related genes. (A) Selective silencing of HIF-1β protein expression by siHIF-1β was confirmed by immunoblot assays. (B) Decreased expression and secretion of EGF, HGF, and VEGF by silencing of HIF-1α and -1β was confirmed by enzyme-linked immunosorbent assay. NT, non-target; siGFP, siRNA against green fluorescent protein; siHIF1-α, siRNA against HIF-1α; siHIF-1β, siRNA against HIF-1β; EGF, epidermal growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor. *, $P < 0.05$.

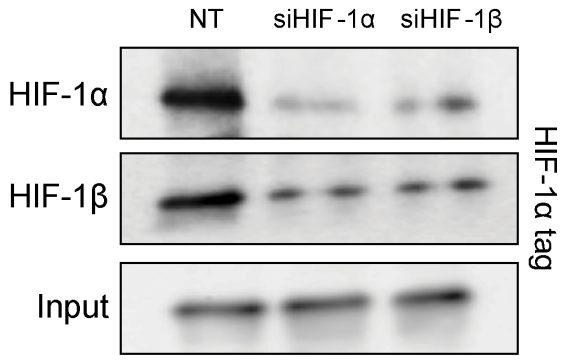


Figure 13. Confirmation of dimerization with HIF-1α and HIF-1β by immunoprecipitation. HIF-1α was bounded with mouse anti-HIF-1α using IgG beads. siHIF-1α or siHIF-1β group weakly detect HIF-1α or HIF-1β band. But, HIF-1α/HIF-1β strongly expressed over 2~3 times in the control group.

4. HIF-1β-silencing suppresses tumor cell invasiveness and motility

Since tumor cells exhibit potential to mobilize and invade into adjacent and distant regions, the effect of HIF-1β-silencing on invasiveness and migration ability of tumor cells was studied using various HCC cell lines. The effect of HIF-1β-silencing on the invasiveness of tumor cells was evaluated by assessing the number of cells that have mobilized and moved across the matrigel-coated trans-well to the gelatin coated-bottom well. Compared to control, siHIF-1β-silenced cells showed remarkably reduced number of cells in the bottom well, denoting suppression of tumor cell invasiveness (Fig 14A). Likewise, as compared to control, markedly diminished migration of HIF-1β-silenced Huh-7 cells was confirmed by scratch and wound healing assay (Fig 14B). Of note, silencing of HIF-1α also induced suppression of tumor cell invasiveness and migration. Together, these findings suggest that silencing of HIF-1β affects the invasiveness and migration of tumor cells under hypoxic conditions.

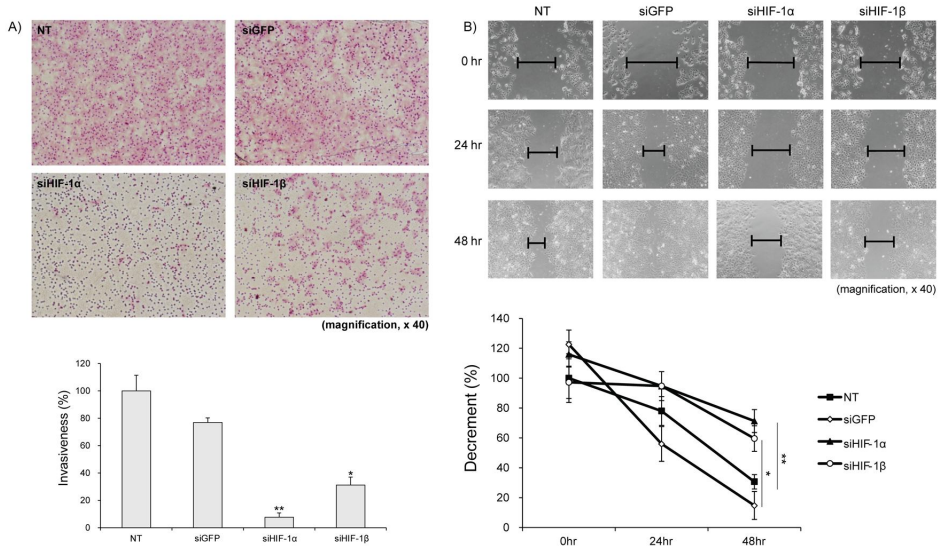


Figure 14. HIF-1β-silencing suppresses tumor cell invasiveness and migration ability. (A) Compared to control, invasiveness of tumor cell was significantly reduced in Hep3B cells transfected with siHIF-1α or -1β as demonstrated by transwell assays. (B) As compared to control, markedly reduced migration of HIF-1α- or -1β-silenced Huh-7 cells was confirmed by scratch and wound healing assay. NT, non-target; siGFP, siRNA against green fluorescent protein; siHIF1-α, siRNA against HIF-1α; siHIF-1β, siRNA against HIF-1β.

5. Silencing of HIF-1β sensitizes tumor cells to hypoxic apoptosis

Normally, cells are predisposed to apoptosis upon exposure to prolonged hypoxic environment, a phenomenon known as hypoxic apoptosis. However, tumor cells tend to circumvent apoptosis through various mechanisms, with the aid of the HIF system. To evaluate whether HIF-1β is responsible for the resistance to hypoxic apoptosis, cell death assay was performed in HCC cells silenced for HIF-1α or HIF-1β followed by exposure to hypoxic environment. Cell death assay demonstrated that hypoxic apoptosis was merely induced in the control (Fig 15). On the contrary, hypoxic apoptosis was markedly increased by silencing HIF-1β, a finding similar to the effect of HIF-1α-silencing on tumor cell survival. Collectively, these data suggest that HIF-1β, along with HIF-1α, regulates hypoxic apoptosis of tumor cells under hypoxic conditions.

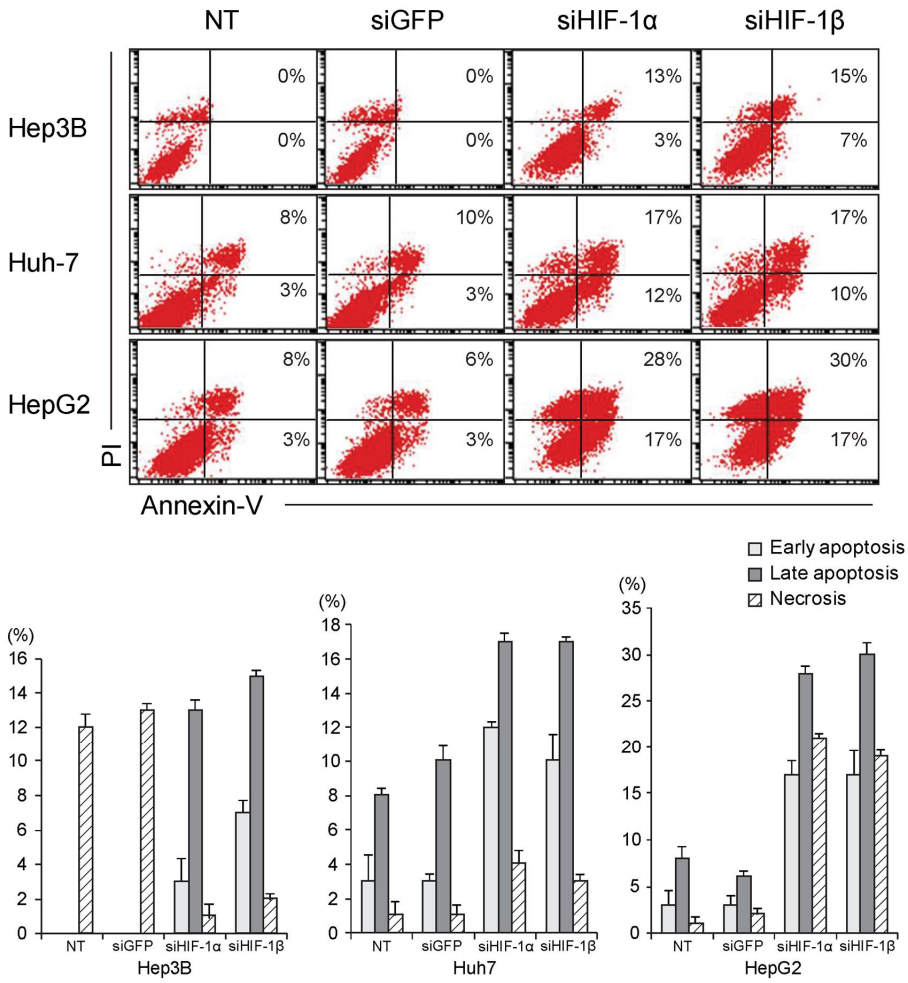


Figure 15. Silencing of HIF-1β sensitizes tumor cells to hypoxic apoptosis. Cell death assay demonstrated that hypoxic apoptosis was markedly increased by silencing HIF-1β in tumor cells, a finding similar to the effect of HIF-1α-silencing on tumor cell survival. NT, non-target; siGFP, siRNA against green fluorescent protein; siHIF1-α, siRNA against HIF-1α; siHIF-1β, siRNA against HIF-1β; PI, propidium iodide.

PART III

Tumor apoptosis induced cell death of vascular endothelial cells by hypoxic stress in hepatocellular carcinoma

1. Silencing of HIF-1α and IL-8 induced hypoxic apoptosis in HCC cell lines

To confirm whether apoptosis was induced by hypoxia induced in HCC cell lines such as Hep3B, the expression of caspase-3, a representative apoptotic factor, and Bcl-xL, an anti-apoptotic factor, were investigated using real-time PCR (Fig 16A). Caspase-3 expression was higher in the HIF-1α-suppressed group than in the control group (Non infection, Ad-shGFP; $p < 0.001$), whereas Bcl-xl expression was inhibited in the HIF-1α-suppressed group. However, the IL-8-suppressed group did not show difference compared to the control group.

Western blot confirmed the PCR results. Expression of the apoptosis factors caspase-3 and caspase-9 was higher in silencing of HIF-1α and IL-8 than those in the control group, whereas there was a decrease in the anti-apoptosis factors Bcl-xl and Bcl-2. Production of intracellular cytochrome c is another way to confirm cell apoptosis (Fig 16B). I confirmed that cytochrome c expression was remarkably increased in the HIF-1α-suppressed group (7000–9000 pg/mL, shHIF, $p < 0.05$; shHIF-1α/shIL-8, $p < 0.001$). However, the IL-8-suppressed group did not differ from the control group in cytochrome c expression. Thus, these results showed increased the expression of various apoptosis factors when the expression of HIF-1α was suppressed.

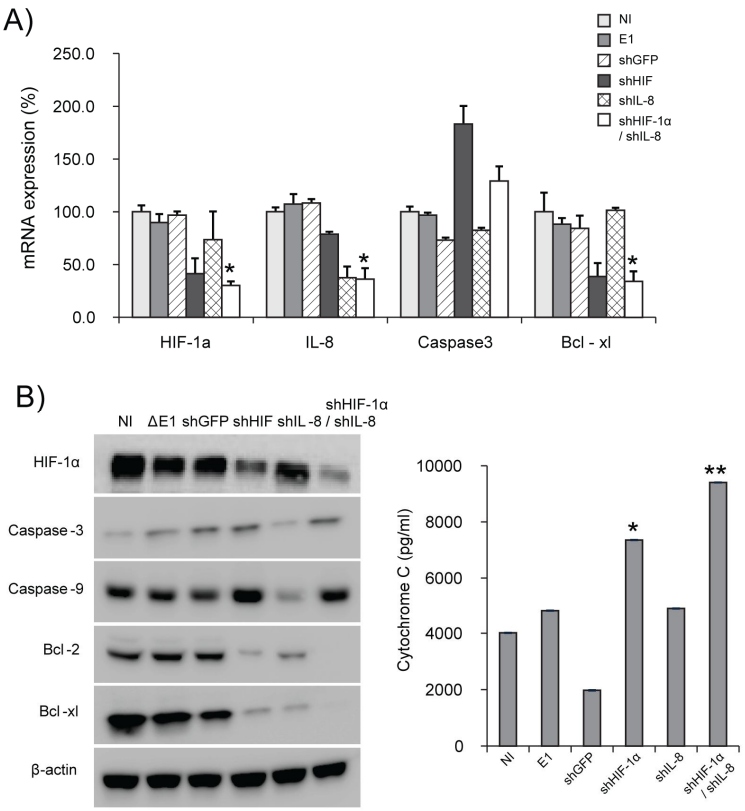
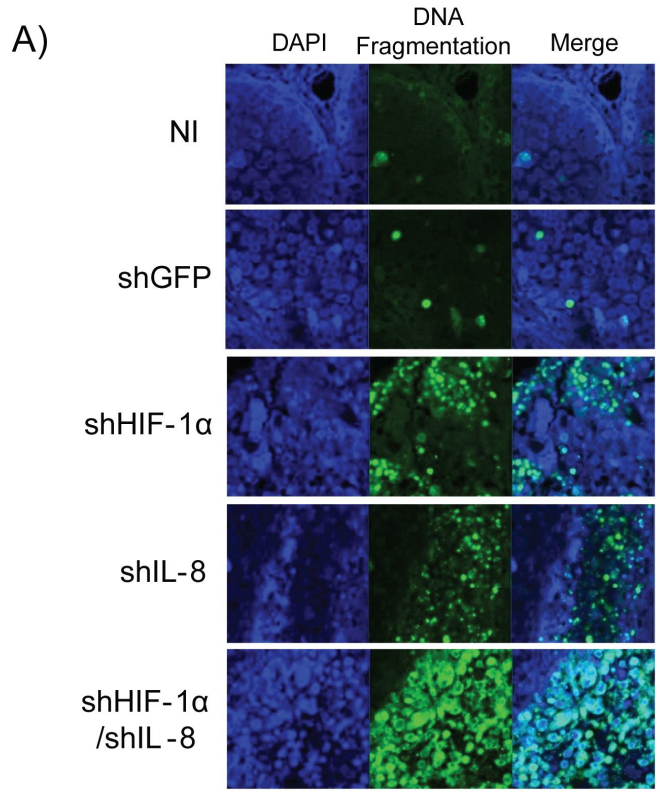


Figure 16. Regulation of apoptosis related factors in Hep3B cells. To confirm the degree of apoptosis, mRNA and protein expression of apoptosis factors were measured. (A) Caspase-3 and Bcl-xl expression was determined through real-time quantitative PCR. Caspase-3 expression increased while Bcl-xl expression decreased in the HIF-1α-suppressed group (Non infection, Ad-shGFP; * $p < 0.05$). (B) Protein expression of relevant apoptosis factors was measured by western blot. The expression of caspase-3 and caspase-9, which induce apoptosis, increased, whereas that of the anti-apoptosis Bcl family members decreased. (C) The expression of intracellular cytochrome c was determined using ELISA. Expression of cytochrome c increased in the HIF-1α-suppressed group (Non infection, Ad-shGFP; * $p < 0.05$, ** $p < 0.001$).

2. Silencing of HIF-1α and IL-8 induced apoptosis in HCC of *in vivo* xenograft model

The effects of inhibition of HIF-1α and IL-8 expression on the apoptosis of carcinoma cell lines were confirmed through aforementioned tumor xenograft models by adenovirus mediated shRNA. In the Hep3B subcutaneous model, tumor tissues were subjected to TUNEL staining (Fig 17A). DNA fragmentation was higher (green fluorescence) in the HIF-1α-suppressed group. The IL-8-suppressed group also showed higher induction of apoptosis compared to the control group.

In addition, the HIF-1α- and IL-8-suppressed group showed higher induction of apoptosis compared to the control group and decreased expression of endothelial markers such as VEGFR2 and Von Willebrand factor [(vWF) VEGFR2, Red; vWF, Orange] (Fig 17B).


A)
DAPI
DNA Fragmentation
Merge
NI
shGFP
shHIF-1α
shIL-8
shHIF-1α /shIL-8

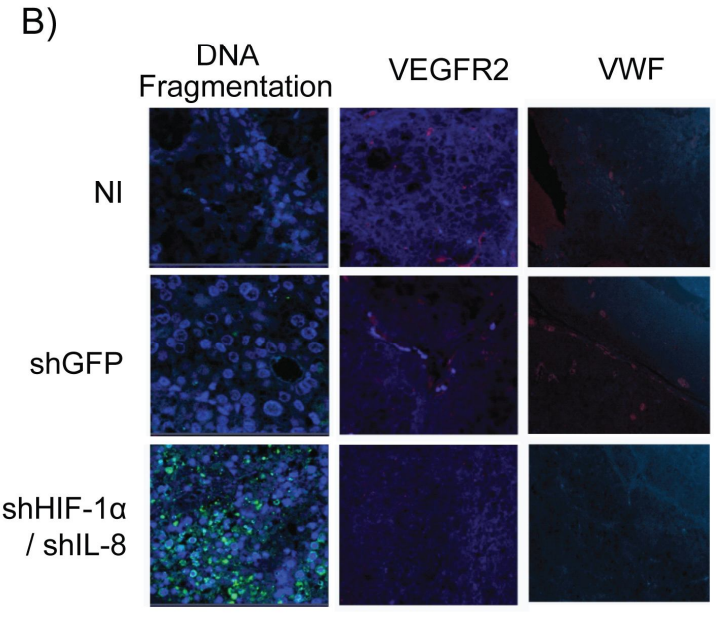

B)
DNA Fragmentation
VEGFR2
VWF
NI
shGFP
shHIF-1α / shIL-8

Figure 17. Apoptosis effects of HIF-1α and IL-8 knockdowns in a tumor xenograft model. DNA fragments in tissues were measured via an in vivo TUNEL assay. (A) Almost no DNA fragments were found in the control group (green fluorescence) whereas DNA fragments increased in the HIF-1α-suppressed group. The IL-8-suppressed group also partially manifested DNA fragments. (B) As DNA fragments enabled measurement of the degree of apoptosis, expression of VEGFR2 and VWF, vascular endothelial markers, were compared. The control group hardly showed any DNA fragments but showed clear expression of VEGFR2 and VWF. On the other hand, the HIF-1α- and IL-8-suppressed group had numerous DNA fragments while exhibiting almost no expression of VEGFR2 and VWF.

3. HUVEC induced apoptosis by conditioned media of apoptosis induced HCC cell lines

FACs analysis was performed to investigate apoptosis in HCC cell lines and vascular endothelial cells. Cells were stained with the TUNEL marker annexinV-FITC, and the necrosis marker propidium iodide and FACs analysis was then employed.

In Hep3B (Fig 18A), I found that the HIF-1α-suppressed group induced increased apoptosis (bottom left square). Conditioned media were produced using the media of cultured Hep3B cells. The Hep3B cells were then treated conditioned media with HUVECs and cultured with the same conditions as Hep3B cells (1% O_2). I confirmed that apoptosis in HUVECs increased when HIF-1α was suppressed in Hep3B. Verification was carried out using another HCC cell line, Huh7 (Fig 18B). Huh7 manifested high induction of apoptosis in the HIF-1α-suppressed group. When conditioned media from HIF-1α suppressed Huh7 was applied to HUVECs, apoptosis was substantially increased. Interestingly, knockdown of IL-8 did not increase apoptosis in HCCs compared to the controls but induced increased apoptosis in hypoxia-induced HUVECs. These findings suggest that HIF-1α expression influences apoptosis in both the HCC cell lines and endothelial cells, whereas IL-8 is associated with regulation of apoptosis in vascular cells without substantial influence on the HCC cell lines.

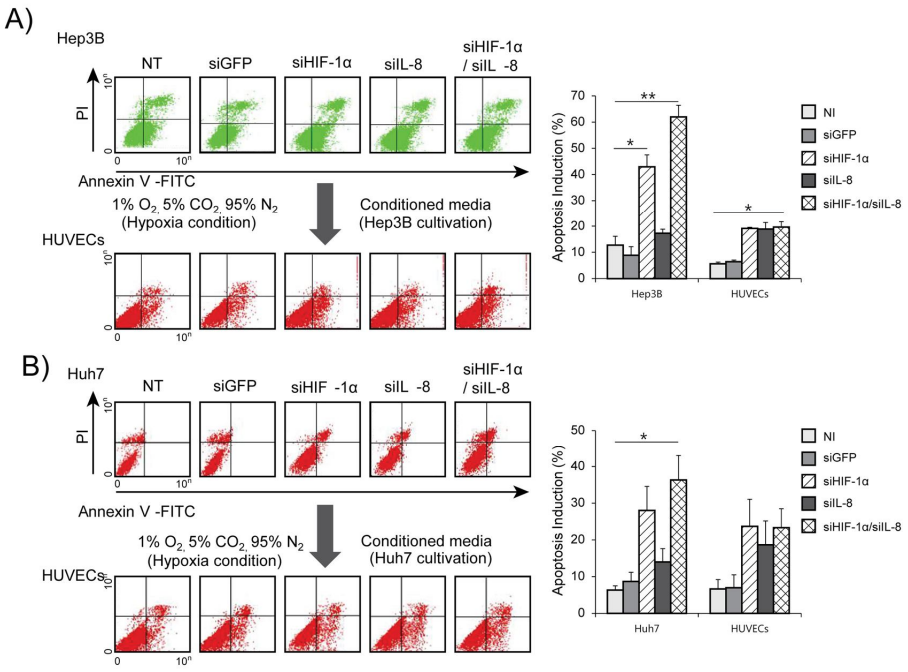


Figure 18. Induction of HUVEC apoptosis by conditioned media of HIF-1α and IL-8 knockdowns in HCC cell lines. Hypoxic stimulation was applied to HCC cell lines, and conditioned media was prepared followed by cross-culturing of HUVECs. They were then cultured under the same hypoxic conditions and apoptosis was confirmed by the generation of DNA fragments. (A) HUVECs were cross-cultured using conditioned media prepared from the Hep3B cell line and hypoxic stimulation was applied. The generation of DNA fragments in the HIF-1α- and IL-8-suppressed group was approximately 3 times more than that in the control (vs siGFP, * $p < 0.05$) . (B) HUVECs were cultured under hypoxic conditions by using conditioned media prepared from the Huh7 cell line. Similar to the pattern with Hep3B cells, increased DNA fragment generation was found in the HIF-1α- and IL-8-suppressed group [(vs siGFP, * $p < 0.05$) approximately 5 times more than that in the control].

4. Silencing of HIF-1α- and IL-8-induced apoptosis of HUVECs cultured in conditioned media

To further investigate apoptosis and, proliferation patterns of HUVECs treated with conditioned media from HIF-1α- and/or IL-8 suppressed Hep3B (or Huh7). Protein expression of apoptotic factors or anti-apoptotic factors were analyzed.

Conditioned media-treated HUVECs were cultured up to 48 h under normal oxygen conditions or hypoxic conditions, and differences in growth over time were examined (Fig 19A). Under both normal conditions and hypoxic conditions, the control group showed increased proliferation in a time-dependent manner; however, time-dependent proliferation was not observed in the HIF-1α- and/or IL-8-suppressed group cultured in conditioned media under hypoxic conditions. Interestingly, under normal oxygen conditions, the IL-8-suppressed group showed greater suppression of growth than that in the other groups. On the basis of the results of a previous study[8], which described that the expression of IL-8 is involved in the proliferation of vascular cells, i were able to confirm that IL-8 had a significant impact on the growth of HUVEC under both normal oxygen conditions and hypoxic conditions.

I observed that the levels of caspase-3, caspase-8, and caspase-9 (Fig 19B), were increased in HUVECs treated with conditioned media from the HIF-1α- and IL-8-suppressed group. Expression of the anti-apoptotic factors Bcl-xl and Bcl-2 was decreased.

The results indicate that suppression of HIF-1α- and IL-8 in HCC cell lines induces apoptosis of vascular cells.

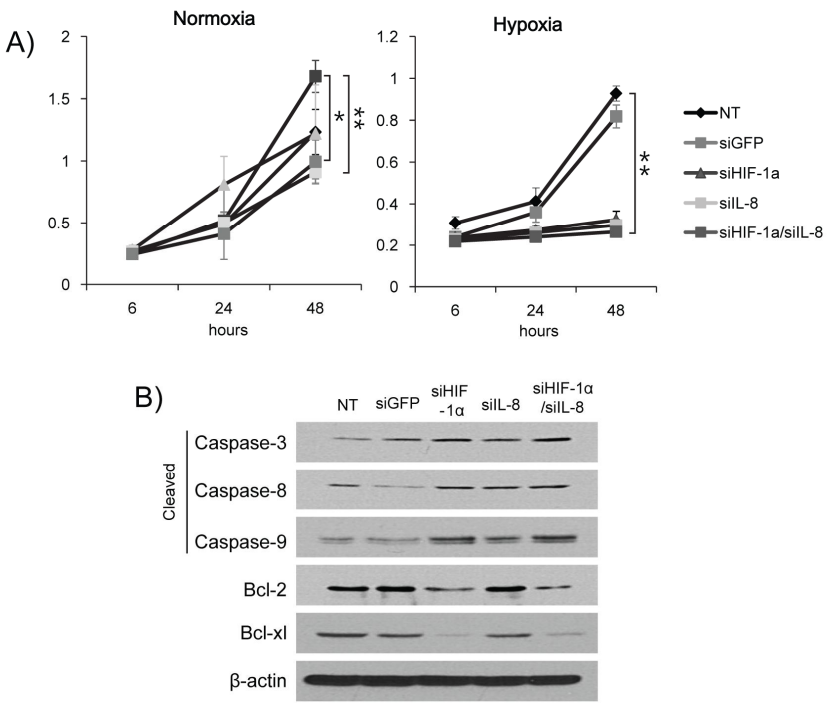


Figure 19. Induction of HUVEC apoptosis in vitro by conditioned media from HCC cell lines. Cell growth and apoptosis related protein expression of HUVECs that were cross-cultured in conditioned media of HIF-1α and IL-8 knockdowns in Hep3B cells were confirmed. (A) Cell growth of HUVECs that were cross-cultured under normal oxygen and hypoxic conditions was determined using the WST-1 method. Mostly, the growth of cells increased over time under normal oxygen conditions, while the growth decreased in the IL-8-suppressed group compared to the control group. Under hypoxic conditions, the HIF-1α- and IL-8-suppressed group showed greater inhibition of proliferation compared to the control group. (B) Expression of apoptotic factors of HUVECs that were cross-cultured under hypoxic conditions was determined. Expression of apoptotic factors increased compared to the control, whereas expression of anti-apoptotic factors decreased.

5. Condition media from HIF-1a and IL-8 knockdowns in Hep3B cells induced time-dependent induction of HUVECs apoptosis under hypoxia condition

Finally, apoptosis patterns over time following hypoxic stimulation were compared to confirm the effects of the stimulation on apoptosis at specific time points.

The shortest hypoxic stimulation of 6 hours and the 24 hours hypoxic stimulation with the highest expression of HIF-1α[5] were compared (Fig 20A). As a result, HCC cell lines showed increased apoptosis over time in the HIF-1α- and IL-8-suppressed group compared to the control; HUVECs did not manifest significant differences in the first 6 hours, but apoptosis largely increased after 24 hours.

To identify the mediators induced by apoptosis of HCC cell lines, which in turn induce the apoptosis of vascular cells, secretion of FasL was measured using ELISA (Fig 20B). At 6 hours, FasL expression was not significantly different compared to that of the control, but apoptosis progressed. With prolonged duration of hypoxia, FasL expression increased compared to the control. On the basis of these results, FasL expression increased as the apoptosis of carcinoma cell lines progressed. Due to such impacts, hypoxic stimulation continuously progressed in the peripheral vascular cells, which lead to the induction of apoptosis.

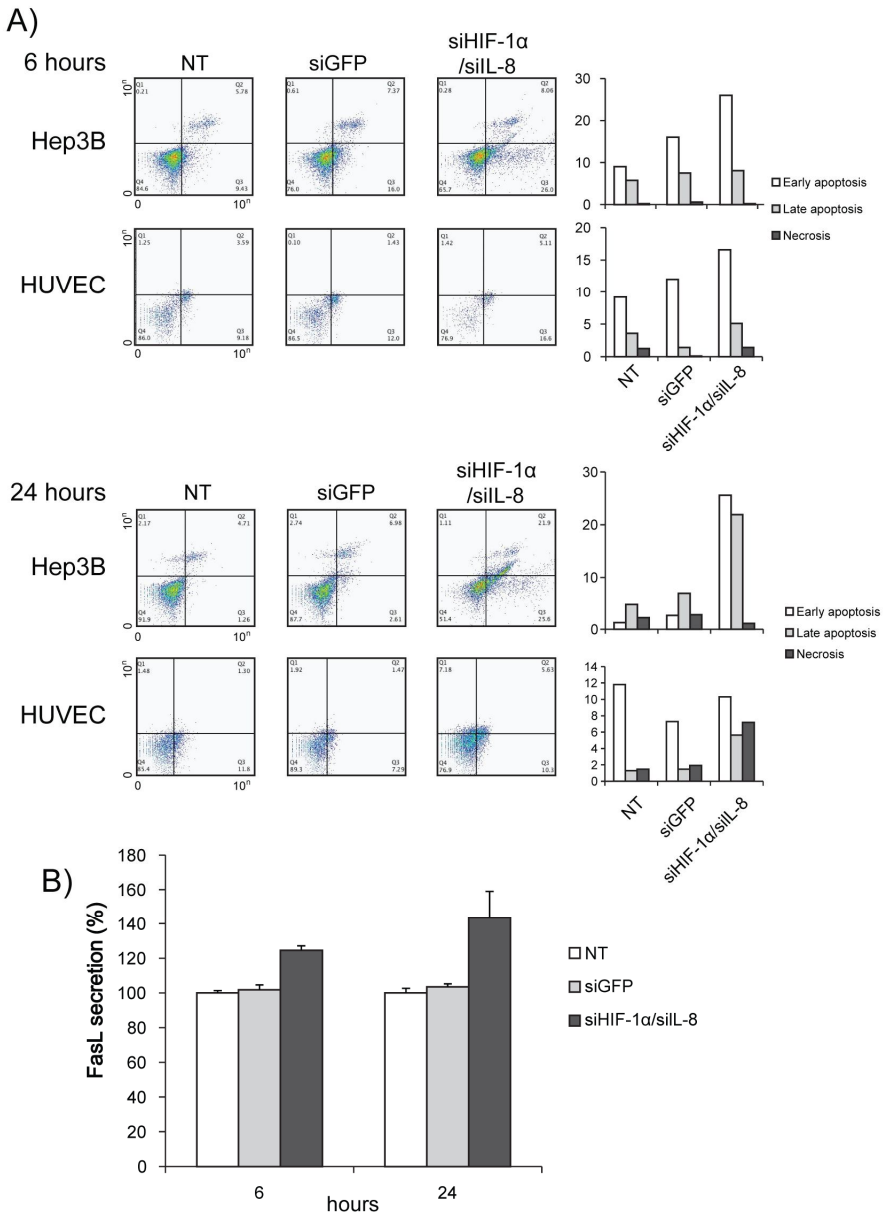

A)
6 hours
NT
siGFP
siHIF-1α
/silL-8
Hep3B
HUVEC
Early apoptosis
Late apoptosis
Necrosis
NT
siGFP
siHIF-1α/silL-8
24 hours
B)
FasL secretion (%)
NT
siGFP
siHIF-1α/silL-8
6
24
hours

Figure 20. Comparison of apoptosis induction between Hep3B cells and HUVECs over time. The degree of apoptosis was compared with respect to the duration of hypoxic stimulation. (A) For the initial 6 hours of stimulation, Hep3B cells were more sensitive to hypoxic stimulation than the HUVECs were, and apoptosis also increased compared to the control. After 24 hours, the apoptosis of HCC cells was induced in the same manner as with the exposure for 6 hours. HUVECs showed an increase in early apoptosis at 6 hours, and late apoptosis also increased at 24 hours compared to the control. (B) In Hep3B cells, expression of FasL, an apoptosis inducer, was investigated over time using ELISA. With 6 hours of hypoxic stimulation, there was an increase compared to the control, but no significant differences. After 24 hours of stimulation, FasL expression manifested remarkable differences compared to the control group.

IV. DISCUSSION

Although newly developed treatments for HCC employ various approaches to combat the disease, all are associated with significant side effects and complications[49,50]. For example, in transcatheter arterial chemoembolization (TACE), which uses vessel embolization to induce cancer tissue necrosis, the surrounding tissue is also obliterated. Furthermore, the remaining, embolization- or radiotherapy-resistant cancer tissue tends to be more malignant and can lead to metastasis[20]. In addition, the hypoxia induced by medical or surgical treatment induces the accumulation of HIF-1α inside tumor cells and its subsequent migration into the nuclei[21], where it promotes the expression of angiogenesis-related genes and increases oxygen supply to the tumor. It also induces the expression of metastasis-related genes[12,22].

These hypoxia-induced processes reduce cellular injury and enable continuous tumor growth by ensuring an effective supply of oxygen for the tumor[23-25]. In recent studies, however, the inhibition of HIF-1α expression failed to block angiogenesis induction by the tumor, allowing the tumor to survive and proliferate[46,47]. The key factor involved in this process is IL-8, which is up-regulated by the hypoxia condition during tumor proliferation. IL-8 induces angiogenesis by activating vascular endothelial cells[9,10,18,23].

In the present study, I investigated whether the simultaneous inhibition of HIF-1α and IL-8 would block the induction of angiogenesis, possibly generating anti-cancer effect[46,47]. An RNA interference tool was used to inhibit the expression of HIF-1α and IL-8[26,51]. Although gene knockdown can be readily achieved by small interfering RNAs (siRNAs), these RNAs are short-lived and thus unsuited for use in animal experiments or in cell proliferation experiments that require a lengthy observation period[48,52]. Therefore, I used RNA interference-inducing adenoviruses for the long-term transient expression of shRNA[53]. Adenovirus infection is a more effective and

efficient means of gene delivery than transfection [53].

A previous study by Mizukami et al. demonstrated that under hypoxic conditions the expression of IL-8 preserves angiogenic response and supports tumor angiogenesis in the absence of HIF-1α [46]. Hence, it could be postulated that tumor angiogenesis can be suppressed effectively by silencing both HIF-1α and IL-8. My results support this hypothesis by demonstrating the inhibition of cancer cell growth by co-silencing of HIF-1α and IL-8 under hypoxic conditions. However, mRNA and protein expression levels of angiogenesis-related proteins showed somewhat confusing results. Although the expression of angiogenesis-related proteins was significantly reduced by co-silencing of HIF-1α and IL-8, silencing of HIF-1α or IL-8 alone also yielded similar results. The underlying mechanism for this functional redundancy in terms of tumor angiogenesis induction by HIF-1α and IL-8 is yet to be determined.

My angiogenesis-related experiments demonstrated phenotypic differences between the knockdown of single gene and that of both HIF-1α and IL-8. Tube formation and microvessel formation were almost absent with the knockdown of both HIF- α and IL-8.

In subcutaneous Hep3B tumor xenograft experiments, there was no significant differences in tumor growth among the groups of HIF-1α knockdown, IL-8 knockdown, and knockdown of both for the first 2 weeks after tumor formation. However, 6 weeks after tumor formation, the group of both knockdowns exhibited more pronounced inhibition of tumor growth than the other single knockdown groups. The group of both knockdowns also showed a higher survival rate.

Immunohistochemical analysis of the excised tumor tissue from the group of both knockdown showed decreased levels of the vascular endothelial cell markers (CD31 and CD34), VEGF-R2 and VE-cadherin. Thus, the anti-angiogenic effect of the suppression of both HIF-1α and IL-8 was clearly evident *in vivo*. In addition, the inhibition of HIF-1α and IL-8 expression also

inhibited the expression of downstream factors favoring angiogenesis and tumor growth, such as VE-cadherin, VEGF-R2 and MMP-2. HIF-1α directly regulated HCC development and IL-8 assisted tumor growth through regulation of angiogenesis in the vascular endothelial systems[17].

Apoptosis also is an important mechanism for the development of organisms, and angiogenesis[28]. Organisms survive and proliferate in the cyclic structure of cell creation and death[57]. However, apoptosis is critical for inhibiting the growth of cancer cells[58]. One of the significant survival mechanisms of cancer cells is the suppression or prevention of apoptosis[37,57,58]. Once apoptosis is induced, cancer cells induce expression of various anti-apoptotic factors, thereby suppressing apoptosis[29,37,56-58]. Thus, the growth of cancer cells or tissues is increased. Various anticancer agents or anticancer therapies have been rapidly developed[39,40]. TAEC, which is currently widely utilized in the treatment of liver cancer[41], induces hypoxia and hypoglycemia in liver cancer cells and reduces the number of cells. Radiation therapies also induce an extended range of hypoxia in the radiated parts[39,41], thereby promoting hypoxic apoptosis of the tissue[28]. Among the various treatments of liver cancer, most methods induce apoptosis of the cancer cells, thereby killing them.

The present study investigated the effects of cancer cell induction or prevention of apoptosis on vascular cells[41,42], one of the peripheral tissues, rather than direct treatment of cancer cells[41]. Apoptosis of cancer cells was confirmed to influence apoptosis or growth of peripheral tissues through various experiments related to apoptosis[38,56]. In particular, angiogenesis of vascular cells, which is closely associated with the growth of cancer cells, also undergoes apoptosis[59,60]. Once apoptosis of cancer cells was induced, the expression of FasL increased in cells and seemed to induce apoptosis in the vascular endotheliaum[43,44]. Although we could not confirm a direct connection between FasL and the Fas receptor in vascular cells, we observed an increase in HUVEC apoptosis when vascular cells were cultured in the conditioned media, with

apoptosis of cancer cells determined via TUNEL-FACs. I also found that the expression of various apoptotic factors increased and that of anti-apoptotic factors was suppressed. The important factor that directly affects liver cancer cells under hypoxic conditions is HIF-1α, although IL-8 is also thought to be a critical survival factor in vascular cells. In the previous studies, IL-8 was shown to be a significant survival and proliferation factor for endothelial cells[61,62], but its roles in the various aspects of cancer treatments are relatively weaker than those for HIF-1α or VEGF[60,63]. Through this study, however, it seems that IL-8 acts as a critical factor in vascular cells and that IL-8 overexpressed in hypoxia is an important target for anticancer agents[41,63].

Cancer cells effectively utilize peripheral tissues for proliferation. Many studies have focused on the growth of the cancer cells and suppression of their proliferation, but there is a lack of studies evaluating the effects of peripheral tissues after suppression of proliferation or death of cancer cells. Tumor preserved in close connection with tumor micro-environment, such as blood vessel. Therefore, regulation of tumor and micro-environment is necessary research of therapy and drug discovery.

In the HCC, hypoxia is an important mechanism that induces proliferation, metastasis, and neovascularization of tumors[65]. HIF-1α depended oxygen concentration and is a key molecule under hypoxia. But, HIF-1β is not affected by oxygen concentration[69]. Although HIF-1α can function by forming a dimer with HIF-1β, a few studies of HIF-1β have been performed[66-69]. HIF-1β was initially known as an ARNT factor, and plays an important role in the differentiation and development of many cell types such as T cells, neurons, and hepatocytes, by dimerization with AhR, which is similar to HIF-1α[69-71]. Moreover, the AhR pathway, which involves HIF-1β (ARNT), is known to mediate anti-tumor effects [72]. Among high-risk patients with HCC in one study, HIF-1β expression was high and was associated with cell cycle arrest [13]. I investigated the function of HIF-1β under hypoxic conditions. The hypoxia

response, which plays a role in tumor development, presumably prioritizes the function of HIF-1β rather than that of ARNT. Accordingly, further study of HIF-1β is warranted.

This study proceeded under the assumption that when dimerization of HIF-1α and HIF-1β is inhibited similar effect will be obtained as direct inhibition of HIF-1α expression. Inhibition of dimerization of HIF-1α and HIF-1β via knockdown of HIF-1β showed similar tumor growth inhibition effects as seen in knockdown of HIF-1α.

Consistent with the effect of tumor inhibition obtained from the MTT assay, expression of the growth factors EGF, HGF, and VEGF related to tumor growth was decreased when determined by real-time quantitative PCR, Western blotting, and ELISA[14]. EGF is a growth factor that stimulates cell growth, proliferation, and differentiation by binding to its receptor EGFR. HGF regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor. VEGF is a signaling protein produced by cells that stimulates neo-vasculogenesis and angiogenesis[13,65]. The above three factors are all related to tumor growth and development, and control of the expression of these growth factors plays an important role in anti-tumor effects. Therefore, expression control of target is another method to inhibit the activation of tumors under hypoxic conditions.

In the present study, the influence of hypoxia on cells and that of HIF-1β knockdown were evaluated using wound healing assay and tumor cell invasion. When HIF-1β expression was inhibited, wound healing was suppressed; similarly, when HIF-1α was knocked down, motility decreased. The current study evaluated the invasion degree of Huh7 cells when expression of HIF-1β was inhibited, and the result was compared with the group for which the expression of HIF-1α was inhibited. When the expression of HIF-1α was inhibited, invasion did not occur as expected. I speculated that inhibition of

HIF-1β expression prevented the formation of a dimer with HIF-1α; in turn, various functions that should be performed under hypoxic conditions, such as inhibited cell survival, cell motility, cell growth, and impeded invasion. Regarding tumor proliferation, hypoxic state is important for tumor growth to start. It is thought that HIF-1 expression (HIF-1α and HIF-1β) controls the initiation of tumor growth, and can be important in affecting anti-tumor growth by changing growth to be more malignant in a hypoxic state. Further study is required to determine other possible functions of HIF-1β that are comparatively less known than those of HIF-1α, which has drawn most of the attention until now.

This findings might be used as a basis for the development of an effective treatment that does not harm normal cells. However, further studies must be conducted before this outcome can be applied clinically. Although the inhibition of HIF-1 and IL-8 had a significant influence on tumor angiogenesis, anti-apoptosis *in vivo* and *in vitro* studies, its effect was restricted to the specific hypoxic condition. Since hypoxia destroys both tumor cells and normal cells, the expression of HIF-1 must be maintained in normal tissues. Also, regulation of tumor or vascular endothelium is necessary field for research of tumor growth and development. After apoptosis of tumor cell or tissue, promoting apoptosis of vascular endothelium prevent recurrence of HCC. In the future, effective cancer treatment could be established by control of HIF-1 and IL-8 expression in cancer-specific hypoxic settings. Therefore, treatment of cancer cells and regulation of vascular endothelium by hypoxia induction could be a useful approach for the development of anticancer agents and treatments.

V. CONCLUSION

HIF-1α directly regulated HCC development and IL-8 assisted tumor growth through regulation of angiogenesis in the vascular endothelial systems. This finding might be used as a basis for the development of an effective treatment that does not harm normal cells. However, further studies must be conducted before this outcome can be applied clinically. Although the inhibition of HIF-1α and IL-8 had a significant influence on tumor angiogenesis in animal studies, its effect was restricted to the specific hypoxic condition. Since hypoxia destroys both tumor cells and normal cells, the expression of HIF-1α must be maintained in normal tissues.

Regarding tumor proliferation, a hypoxic state is important for tumor growth to start. It is thought that HIF-1 expression (HIF-1α and HIF-1β) controls the initiation of tumor growth, and can be important in affecting anti-tumor growth by changing growth to be more malignant in a hypoxic state. Further study is required to determine other possible functions of HIF-1β that are comparatively less known than those of HIF-1α, which has drawn most of the attention until now.

Various treatments have been developed for cancer, and better therapies have been developed by overcoming the limitations of already developed treatments. We hypothesized that if the symptoms that occur during tumor treatment can be studied and controlled, the obstacles currently encountered during cancer treatment can be eliminated. If simultaneous regulation of tumor development, hypoxia, and angiogenesis is possible, cancer cells could be easily treated without peripheral damage. In other words, simultaneous inhibition of the factors that potentially control hypoxia and angiogenesis during treatment to induce apoptosis may be a more innovative anticancer treatment modality.

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ABSTRACT (IN KOREAN)

간암에서의 저산소 조절 과 항종양 효과 연구

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최 성 훈

저 산소는 종양의 과 증식을 통해 산소가 부족하였을 때 나타나는 현상으로 간암에서의 종양발달에 중요한 메커니즘이다. 저산소유도인자(HIF)는 산소부족으로 인한 자극을 통해 발현되며, 혈관신생작용, 세포사 그리고 대사와 관련된 유전자의 발현을 조절하는 중요한 전사활성인자이다. 인터루킨-8 (IL-8) 역시 내피세포의 생존과 혈관신생작용을 조절하는 중요한 인자로 알려져 있다. HIF-1α 와 IL-8의 동시 조절 혹은 HIF-1β의 조절은 지금까지 간암 연구에서 명확하게 연구결과가 밝혀지지 못하고 있다. 그러므로, 본 연구는 HIF과 IL-8의 발현 억제를 통해 간암에서의 혈관신생작용과 세포사 그리고, 종양의 성장에 대해 긍정적인 연구 결과를 도출 할 수 있을 것이다.

간암세포주에 아데노바이러스 매개의 작은 헤어핀 구조를 갖는 HIF-1α와 IL-8을 감염시키고, 저산소 조건(1% O_2, 24시간)에서 배양하였다. 그리고, HIF-1α, IL-8 그리고 혈관신생작용과 세포사에 관여하는 인자의 발현을 면역반응을 사용한 실험기법과 정량적 연쇄중합반응을 이용하여 확인하였다. 또한, 종양의 증식을 MTT 기법을 통해 확인하였다. 면역 형광기법과 세포사 분석 기법(TUNLE-FACs)을 통

해 간암의 세포사와 혈관내피세포의 세포사의 발생을 비교 분석하였으며, 종양세포이식기법을 통해 HIF-1α와 IL-8의 발현을 억제하였을 때 혈관신생과 세포사 관여하는 인자의 발현 양상을 비교 분석하였다.

HIF-1α의 발현 억제는 직접적으로 종양의 증식에 영향을 주며, IL-8은 간접적으로 종양의 증식을 저해하였다. *In vivo*에서 HIF-1α와 IL-8의 동시억제는 생존률을 증가시키고 종양의 부피를 감소시킨다. 또한 혈관생성 밀도를 감소 시키며, 혈관신생작용을 확인 할 수 있는 침윤과 관형성의 저해를 유도화하였다. 또한, HIF-1α와 IL-8의 발현억제는 세포사 유도 인자의 발현을 증가시키고, 항세포사 유도인자의 발현을 감소시켰다. HIF-1α와 IL-8의 발현억제는 세포내의 cytochrome C의 농축을 증가시키고, DNA의 단편화를 증가시킨다. 또한 세포 배양액을 통해 전달 된 세포사 유도인자를 통해 혈관내피세포의 세포사 역시 증가시킨다. 그리고, 저산소 조건에서, HIF-1β의 발현억제는 HIF-1α와 비슷한 수준으로 종양 증식의 억제를 유도하여, 종양증식인자들의 발현을 억제하였다. 또한, 종양세포의 이동과 침윤을 저해하며, 세포사의 유도를 통해 항종양 효과를 증대시킨다.

작은 헤어핀 구조를 통해 HIF-1 과 IL-8의 발현 억제는 간암에서 종양의 혈관신생작용과 증식 그리고 항 세포사를 억제하였다. 그리고 혈과내피세포의 증식과 혈관내피세포의 세포사를 통해 항종양 효과를 확인 할 수 있다. 게다가 HIF-1 와 IL-8을 인지하는 작은 헤어핀 구조를 갖는 RNA 간섭의 발달은 간암의 치료에 중요한 치료 기법으로 적용 할 수 있을 것이다.

저산소 조건을 조절 하는 것은 매우 중요한 치료방법이 될 것으로 생각된다. 정상적인 세포와 암세포의 가장 큰 차이점은 많은 영양소와 산소를 소모하여 증식과 분열에 대부분의 에너지를 소모한다. 그

결과 종양 조직은 국소적으로 저산소 상태가 나타나며, 이러한 부분을 표적으로 한 치료방법은 정상세포에는 크게 악영향을 주지 않는 치료법으로 유용할 것이다. 또한, 지금까지 HIF-1α와 VEGF등에 초점이 맞추어져 있는 치료법이 대두 되었지만, IL-8등의 좀더 세포독성이 덜한 표적의 치료방식은 효과적으로 암세포를 제거하고 치료 역시 용이할 것으로 생각된다.

핵심되는 말: 저산소, 종양증식, 혈관신생, 세포사, 저산소유도인자-1α와 β, IL-8, 항종양 효과, 아데노바이러스매개의 작은 헤어핀구조

PUBLICATION LIST

1. **Choi SH**, Shin HW, Park JY, Yoo JY, Kim do Y, Ro WS, Yun CO, Han KH. Effects of the knockdown of hypoxia inducible factor-1α expression by adenovirus-mediated shRNA on angiogenesis and tumor growth in hepatocellular carcinoma cell lines. The Korean Journal of Hepatology. 2010 Sep;16(3):280-7. (Award for Best Journal in KASL)

2. **Choi SH**, Kwon OJ, Park JY, Kim do Y, Ahn SH, Kim SU, Ro SW, Kim KS, Park JH, Kim S, Yun CO, Han KH. Inhibition of tumour angiogenesis and growth by small hairpin HIF-1α and IL-8 in hepatocellular carcinoma. Liver Int. 2014 Apr;34(4):632-642

3. **Choi SH**, Chung AR, Kang W, Park JY, Lee MS, Hwang SW, Kim DY, Kim SU, Ahn SH, Kim S, Han K. Silencing of hypoxia-inducible factor-1β induces anti-tumor effects in hepatoma cell lines under tumor hypoxia. PLoS One. 2014 Jul 28;9(7):e103304.